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(54) Title: SOLID SUPPORT FOR USE IN CELL CULTIVATION, ESPECIALLY FOR THE CULTIVATION OF LIVER CELLS, BIOLOGICAL REACTOR CONTAINING SAID SOLID SUPPORT AND THE USE THEREOF IN A BIO-ARTIFICIAL LIVER SYSTEM

(57) Abstract

The invention relates to a solid support for use in cell cultivation in vitro, comprising a 3D-matrix material and hollow fibres being permeable to at least gaseous oxygen and/or gaseous carbon dioxide, a biological reactor for the cultivation and/or maintenance of living cells comprising said solid support, as well as a method for culturing and/or maintaining living cells, using said solid support and/or said biological reactor. The solid support, biological reactor and method are especially suited for culturing human or animal derived liver cells, for use in or as a bio-artificial liver. The invention further relates to such a bio-artificial liver, as well as a method for treating liver disorders using said bio-artificial liver.

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Solid support for use in cell cultivation, especially for the cultivation of liver cells, biological reactor containing said solid support and the use thereof in a bio-artificial liver system.

Field of the invention

The present invention relates to the field of the cultivation of cells, especially of adherent tissue cells such as liver cells. More in particular, the invention relates to the field of biological methods and reactors for the cultivation and/or maintenance of cells, especially liver cells, and to the use of such methods in a bio-artificial liver system (BAL).

Brief description of the prior art

It is generally known that most tissue cells require a solid support on which to grow and divide.

Although it is possible to culture adherent tissue cells in ordinary vessels, such as glass bottles or Petri dishes, during which the cells adhere to the wall of the vessel, usually special reaction vessels or bottles with a high surface area are used so as to provide increased capacity for cell attachment. One way to improve said surface area is to use a solid support for cell adherence. Such solid supports are known in the art; examples include glass beads, microcarriers and cellulose fibres.

A special problem in the cultivation of adherent cells - compared to the cultivation of cells in suspension or in confluent layers - is to provide sufficient nutrients and/or oxygen to the cells and/or provide for sufficient removal of waste products and/or carbon dioxide. This is especially a problem with cells that put stringent demands on both oxygenation as well as the removal of waste products, such as liver cells.

The non-availability of suitable solid supports and methods for the in vitro cultivation of liver cells has

over the last 40 years severely hindered the development of the so-called bio-artificial liver (BAL) systems, systems that could be used in patients with liver defects for the support and/or replacement of the natural liver function. As acute liver failure has a very poor prognosis and is usually fatal to the patient within days or even hours [vide for instance Devlin et al., Hepatology Vol. 21, No. 4 (1995), pages 1018-1024 and Lake and Suzman, Hepatology, Vol. 21, No. 3 (1995), pages 879-882, describing the general problems in the art of the treatment of liver failure, both incorporated herein by reference], because livers for transplant are not readily available, a BAL system that could support and/or replace liver function, for instance during the time the patient awaits for a liver to become available for transplant and/or to bridge the period until the liver of the patient sufficiently recovers and/or regenerates by itself and/or as a result of treatment, would be highly desirable.

However, due to the abovementioned lack of suitable methods and/or materials for cultivating and/or maintaining liver cells in vitro, the bio-artificial liver systems from the prior art have so far proved insufficient, because they do not fully replace all the functions carried out by the liver of the patient in vivo, because they have insufficient capacity, and/or because the time during which they are therapeutically effective is too limited for practical use.

The history of bio-artificial liver systems has been described in a number of recent articles, notably Nyberg et al., the American Journal of Surgery, Vol. 166, November 1993, p. 512-521, and Suzman and Kelly, Scientific American, May-June 1995, p. 59-77, incorporated herein by reference.

As described in these articles, the earliest liver support systems were based on haemodialysis, charcoal hemoperfusion, or cross-haemodialysis either between

humans or between humans and animals. Also, extra-corporeal liver perfusion has been tried.

All these systems have been found to be insufficient. As stated by Nyberg et al.:

"based on the limited success achieved by early liver support techniques, the concept evolved that liver functions essential for survival would be best provided by mammalian liver preparations that allowed sustained or repetitive application. These liver preparations, commonly referred to as hybrid or bio-artificial systems, contain biological components within a synthetic framework. Biological components may include isolated liver enzymes, cellular components, slides of liver or cultured hepatocytes. Hepatocytes may be implanted in the patient or perfused extra-corporally. Hepatocytes systems have shown the greatest promise for bio-artificial liver support. When compared with cellular component and isolated enzyme systems, hepatocyte systems should supply a greater number of liver functions, since they utilize intact, metabolically active liver cells (....). One major advantage of the hepatocyte bio-artificial liver over traditional hepatocyte transplantation and earlier support techniques, such as cross-circulation and extra-corporal liver perfusion, is that the bio-artificial liver can be constructed from semipermeable materials that provide a barrier between the hepatocytes and the host immune system. As a result bio-artificial liver therapy may be performed without immunosuppression, and hepatocytes from different species (xenocytes) may be used within the bio-artificial liver.

The disadvantages of bio-artificial liver systems include (...) the problem of maintaining normal hepatocyte viability and function at the high cell density necessary for clinical application. For

example, when hepatocytes are grown on a plastic surface with standard cell culture medium, they lose their gap junctions in about 12 to 24 hours: they also flatten and become a granular: tissue specific functions are lost in 3 to 5 days, followed by hepatocyte death within 1 to 2 weeks. As a result, improved techniques of cell culture have become necessary for the application of bio-artificial liver support systems."

A number of different approaches to the cultivation of hepatocytes and related cells for use in or as BAL-systems have been described. However, the prior art hepatocyte systems also suffer from problems with regard to capacity and effective working time, vide Sussman and Kelly:

"With regard to the provision of sufficient metabolic capacity, it is not clear exactly how much liver necrosis is fatal. Animal experiments suggest that at least 30% of the liver's original function must be preserved in order to survive. The adult human liver contains approximately 1000 gm of hepatocytes, which are the metabolically active cells. Thus we have proposed that effective liver assistance will require the equivalent of 300 to 400 gm of cells.

Two sources of hepatocytes are available: freshly isolated cells (primary cultures) and cells grown in continuous culture (cloned or immortalized cells). Cells that have been isolated from a normal human or animal liver retain many of their functions (....) the technology has severe limitations. Artificial livers that use freshly isolated cells have so far provided only a fraction of the necessary metabolic capacity. Hepatocytes do not divide after they have been isolated, so a steady supply of new cells is required. Coupled with the labour-intensive nature of cell preparation, this makes it

almost impossible to scale up production to meet current needs in a cost-effective manner. Moreover, freshly isolated cells do not appear to last very long during treatment. A liver assist device that lasts for only 6 to 7 hours, as some have been reported to do, clearly falls short of allowing liver regeneration. Finally, production of any such device using animal cells entails a number of problems, especially in areas of sterility and lot-to-lot variability."

Uchino et al, ASIAO Transactions 1988;23;972-977 describe a hybrid bio-artificial liver composed of multiplated hepatocyte monolayers. A total of 80 grams of cultured adult dog hepatocytes was cultured in a reactor comprising a stack of 200 collagen coated borosilicated glass plates. These hepatocytes were viable and functioned well during 4 weeks in perfusion culture. This bio-artificial liver was tested in anhepatic dogs. The longest survival obtained was 65 hours.

However, a serious drawback of this system, besides the complexity of constructing and using a 200 glass plate-reactor, is that the monolayer culture of hepatocytes on said plates precludes the advantageous formation of hepatocyte aggregrates. It is well known in the art that hepatocytes cultured in or as aggregrates function both longer and better than hepatocytes cultured in monolayers, showing higher activity and better differentiation.

Another approach in the development of bio-artificial liver systems has been the use of hollow fibre bioreactors in which liver cells are present in the extra-fibre (extraluminar) space while a liquid medium is pumped through the fibre lumen (intraluminar space), usually by perfusion with whole blood or plasma.

Rozga, Demetriou et al. Biotechnology and Bioengineering, Vol. 43 (1994), incorporated herein by reference, give an overview of the current hollow fibre

systems. Their own system consists of a high flow plasma perfusion circuit comprising a charcoal column and a porous hollow fibre module with 5 to 6 x 10^9 microcarrier-attached porcine hepatocytes seeded into the extra fibre compartment. Because of the use of solid support (collagen coated dextran microcarriers), the surface area available for hepatocyte attachment is increased.

However, this design requires a separate membrane oxygenator for the oxygenation of the plasma to be incorporated into the perfusion circuit so as to provide sufficient oxygen to the hepatocytes in the hollow fibre module. Therefore, said oxygenation as well as the removal of carbon dioxide are dependent upon limiting factors such as the solubility of oxygen and carbon dioxide in the plasma and the transport of the oxygenated plasma throughout the reactor. Because of these limitations said hollow fibre reactor cannot easily be scaled up to a capacity required for practical therapeutical application.

Furthermore, this reactor is used with a very "closed path" column with a high density of the macrocarriers, which leads to the formation of microcarrier pellets and to mass transfer problems with regard to the cells at the center of such a pellet.

Another disadvantage of this system is that the hepatocytes first have to be immobilised on the microcarrier before the hepatocytes can be introduced into the hollow fibre reactor. This involves further complicating processing steps that can lead to loss of cell viability.

Sussman and Kelly, mentioned hereinabove, describe a hollow fibre-based bio-artificial liver system in which liver cells are attached to capillaries through which whole blood from the patient is pumped.

According to this system, the liver cells are oxygenated by the patients blood, because -as stated by the authors- "plasma does not provide the oxygen carrying capacity of whole blood".

Furthermore, perfusion with whole blood can lead to the fibres and/or the pores thereof within the bioreactor getting clogged, which problem could only be solved by totally replacing the hollow fibre module, requiring a fresh isolation/immobilisation of the hepatocytes.

Other disadvantages of this and other hollow fibre systems using whole blood as the liquid medium are that "the hollow fibre membrane must first act as a plasma separator before any significant transport of nutrients and metabolites can take place across the fibre wall", and that it "requires systemic anticoagulation with heparin to prevent clotting in the module".

Also, in order to overcome problems with the isolation of cells, in this BAL-system a special cell line named C3A derived from a liver tumour of a child is used. However, with regard to activity and function, the use of such tumour-derived cell lines is generally less preferred in the art than the use of isolated primary hepatocytes, also from a safety standpoint.

Furthermore, the C3A cell line used by the Suzman lacks some very important functions of primary hepatocytes. Also, the C3A cells are less differentiated, and therefore less active than primary liver cells.

A somewhat different hollow fibre system is described by Nyberg et al, mentioned hereinabove: hepatocytes are suspended in a collagen gel, which is injected into the lumen of hollow fibres. After that, the extra fibre space of the bioreactor is perfused with medium for 24 hours, after which the gel contracts within the fibres, thereby creating a third space which is perfused with medium.

The idea behind this three-compartment design is that blood can be pressed through the extra fibre compartment, whereas the gel entrapped cells are nourished and possibly stimulated by the factors present in the medium flowing through a path adjacent to the contracted collagen.

However, this system also requires a complicated and time consuming pre-immobilisation of the hepatocytes.

Another BAL-system based on capillaries for hepatocyte immobilization is described by Gerlach et al., Transplantation, Vol. 58, No. 9 (1994). Their bioreactor consists of a three dimensional framework for decentralized cell perfusion with low metabolite gradients and decentralized oxygenation and CO_2-removal, consisting a woven network of four discrete capillary membrane systems, each serving different purposes, i.e. I, plasma inflow (polyamide fibres); II, oxygenation and carbon dioxide removal (hydrophobic polypropylene fibres or silicon fibres); III, plasma outflow (polysulfon fibres); and IV, sinusoidal endothelial coculture (hydrophilic polypropylene fibres). These capillaries must be woven in such a way that the majority of hepatocytes find all four types of membranes in their surroundings.

This reactor was used with 2.5×10^9 pig hepatocytes with a viability between 88 and 96 %, which were co-cultured with autologous sinusoidal endothelial cells present in the co-cultured compartment of the reactor.

In this type of hollow fibre bioreactor the liver cells have to be attached directly to the hollow fibres as no further matrix material for cell attachment is present in the reactor. In order to obtain sufficient attachment of the cells, the surfaces of the fibres must first be coated with a proteineous basement membrane product, such as Matrigel^R or other collagen-based materials, requiring a separate and expensive pretreatment step. Even so, as hollow fibres are not specifically designed and/or suited for use as a solid support in cell cultivation, the attachment and the speed thereof permitted by and/or obtainable with said reactors is limited and heavy inoculum charges are required when seeding the reactor.

Furthermore, the average fibre distance within said threedimensional fibre framework is about 500 µm, leading

to the formation of large cell aggregates of comparable size. Again, these large aggregates can lead to mass transfer problems with regard to the cells in the centre of said aggregate.

Also, it is well known that hollow fibres are difficult to process, and in this respect the manufacture of the very complicated three-dimensional fibre network described by Gerlach et al., comprising four separate discrete capillary systems, suffers from a disadvantage from an economical point of view. Also, this reactor is complicated to operate, requiring multiple separated inlet/outlet control systems.

A general problem of all the abovementioned hollow fibre bioreactors of the prior art is that the liquid medium (blood, plasma) to be treated is separated from the hepatocytes by the hollow fibre membrane; in other words, that there is no direct contact between the liquid medium and the hepatocytes in the reactor. Nutrients and substances to be removed from the liquid medium and/or to be secreted into into the liquid medium, have to pass through said membrane barrier in order to reach the hepatocytes and the liquid medium, respectively. The passage through the membrane can lead to transport phenomena that can limit the achievable mass transfer, and therefore the efficiency of the BAL-system.

Also, the membranes can get clogged, especially when perfusion with whole blood is used. In that case the BAL system or parts thereof have to be replaced, which means that therapy has to be interrupted or even stopped.

Another important limiting factor in the membrane transport is the molecular weight cut off of the membrane, vide Nyberg et al:

> "Permeability and membrane molecular weight cut off influence waste removal, product delivery, and immune activation. Performance of biotransformation functions and the removal of nitrogenous wastes are important functions of the bio-artificial liver,

along with the removal of red blood cell breakdown products such as bilirubin. The production of coagulation proteins and other serum proteins by hepatocytes in the bio-artificial liver may also be beneficial to patients with liver failure. However, these proteins are of comparable sizes to antibodies, which could have an adverse effect when directed against nonautologous hepatocytes in the bioreactor. Alternatively, small peptide products of the hepatocytes may exit the bioreactor and serve as antigenic stimulant in the patient. Whether these foreign molecules will result in harmful cytokine production, immune complex formation, or serum sickness in patients with liver failure remains to be determined. Potential side-effects must be addressed experimentally in order to determine the best molecular weight cut off for use in the bio-artificial liver.

Clinical treatment of hepatic failure requires large scale, high density hepatocyte culturing. In many bioreactors this gives rise to the formation of non-physiological hepatocyte pellets. Hepatocytes in the center of these large aggregates show poor metabolic activity and even possible necrosis due to high gradients as a result of hindered transport of nutrients and oxygen to and carbon dioxide, toxins and cell products from these cells. This is in contrast to the in vivo liver where every hepatocyte is in close contact with the blood. Besides, in most systems substrate exchange depends on diffusion which further limits mass transfer compared to the in vivo situation where hepatocytes function under perfusion conditions with low gradients.

Also, the bioreactors of the prior art are limited with respect to the amount of liquid medium that can be withdrawn from the hollow fibre lumen, as in general the fusion transport will be too slow. Therefore, an active withdrawal of liquid medium from within the hollow fibres

will be required, even so, the total flow through the hollow fibre membrane will be very slow and/or lead to the undesired formation of gradients, even with a high flow of liquid medium through the hollow fibres themselves.

Another general problem with the bio-artificial liver systems of the prior art is that they require the use of liver cell preparations with a high viability (> 80 %) and a high attachment. As already acknowledged by Sussman and Kelly hereinabove, the production of such cells is a very costly, complex and time-consuming process requiring isolation and subsequent cultivation of suitable liver cells in sufficient viability and quantity which involves complicated procedures that do not reliably afford the required results, even when carried out by qualified experts.

Furthermore, known hepatocyt-containing BAL-systems cannot be stored before use for a prolonged period of time because the viability and function of the liver cells in the reactor cannot be maintained at a therapeutically acceptable level.

Also, the only technique available for preserving isolated liver cells over a longer period of time, i.e. cryo-preservation, does not afford cells that are suitable for use with known BAL-systems, vide Rozga et al, mentioned hereinabove:

> "availability of cells on demand becomes a very important consideration in the clinical setting where treatment of patients with FHF is carried out emergently, on short notice and at all hours. However, [cryopreservation] may result in a significant loss of cell viability [...] and attachment (as much as 50 %). [....] Therefore, in clinical settings, we prefer the use of freshly isolated, well attached hepatocytes."

Because of these problems, the known BAL-systems cannot be used as "off the shelf" units that can be kept

and/or maintained in hospitals until their use is required, as is the case with other artificial systems for organ support such as for instance dialysis machines or artificial heart or long systems. Also, replacement during therapy of a spent primary liver cell based BAL system of the prior art with insufficient function with a fresh BAL system is usually not economically feasible over a prolonged period of time.

For instance, Demetriou reports that after 6 hours of use, 50 % of the primary liver cells within his reactor die, whereas within 24 hours all cells have died. Better results have been obtained by using immortilized cells or the C3A cell line reported by Sussman et al., however, the use of this hepatoblast derived cell line has other disadvantages as already mentioned hereinabove.

In view of the above, there is a continuing need for bio-artificial liver systems that do not have the abovementioned disadvantages of the prior art systems.

The British patent application 2,178,447 describes a matrix for cell cultivation in vitro providing an increased available effective surface area for cell attachment provided by a fibre network or open-pore foam with a suitable pore size 10 µm to 100 µm. This matrix material can be provided in the form of a sheet or mat or in the form of particles or flakes, in which latter form it is marketed by Bibby Sterilin under the name Fibra-Cel ®. As a sheet or mat, this matrix material has an appearance like filter paper or tissue paper, or thin porous felt.

This matrix material has some specific advantages over micro-capsules, which are costly and delicate to produce and give problems at high cell density growth, because frequently cells at the centre of the capsule die. Also, the microcapsules may burst prematurely losing their contents and each new inoculation requires a fresh encapsulating procedure. Compared to microcarriers the matrix material according to GB-A-2,178,447 has the

advantage that the cells are immobilized within the matrix structure. With microcarriers, these cells are immobilized on the outside of the carrier particles, making them susceptible to shear stress and particle collisions, for instance during preparation or packing of the reactor.

Furthermore, in the matrix material according to GB-A-2,178,447, the cells can proliferate along the fibres of the sheet in three dimensions (3D), rather than in two dimensions as in conventional tissue culture bottles, flasks or Petri dishes or on microcarrier beads or hollow fibres. Cells may attach themselves to more than one fibre and cell growth takes place in the internal volume of the fibre matrix. For these reasons, this and similar matrix materials are known in the art as "3D-carrier matrices".

Another advantage of said 3D-matrix material is that it does not require the heavy inoculum charges of two dimensional systems (20-30 % of the final amount of cells at saturation), but can be inoculated at amounts of less than 10%, and as low as 5%. The three dimensional network provides for a higher - and quicker - "capture" of the cells, thereby also making it possible to use cells with sub-optimal attachment.

The GB-A-2,178,447 furthermore describes a number of potential bioreactor geometries employing the matrix material described therein. One of these comprises a sheet of said matrix material, rolled up into a spiral between two flattened tubes, wherein each alternate flattened tube serves a conduit, one for liquid nutrient medium, and the other for gases such as oxygen, air, CO_2 and water vapour.

However, GB-A-2,178,447 is not directed to the construction of bio-artificial liver systems, nor to the specific problems relating to the cultivation and/or maintenance of hepatocytes therein. In particular,

GB-A-2,178,447 does not relate to the special problem of supplying sufficient oxygen to highly oxygen dependant hepatocytes.

In fact, the use of the "spiral wound" reactor according to GB-A-2,178,447 for culturing and/or maintaining hepatocytes would in practice lead to insufficient oxygenation, because the oxygen is supplied by means of just one conduit covering the entire lenght of the matrix mat. The use of such a single conduit would lead to the generation of an undesired oxygen gradient along its length or even to local oxygen depletion, especially when the reactor is scaled up by increasing the number of matrix windings.

Also, the bioreactor construction according to GB-A-2,178,447 contemplates a separate conduit for the supply and/or removal of the liquid medium, so that during use as a BAL, nutrients, toxins and other substances to be absorbed or secreted would have to pass through the membrane surrounding said conduit in order to reach the hepatocytes, giving the problems with regard to membrane transport and mass transfer as described hereinabove.

Furthermore, the use of a singular spiral wound conduit for liquid transport can lead to an inhomogeneous supply of liquid medium to all the parts of the bioreactor, for instance by the generation of undesired gradients.

All these factors make the matrix material as such and the bioreactor according to GB-A-2,178,447 unsuited for use in the cultivation of liver cells and/or for use as a BAL.

Objects of the invention

It is therefore a first object of the invention to provide an improved solid support and bioreactor for the cultivation and/or maintenance of adherent cells, especially liver cells, with improved cell adherence pro-

perties and improved supply and/or removal of gaseous components such as oxygen and carbon dioxide, even when used in or as a large scale bioreactor.

It is a further object of the invention to provide an improved solid support and bioreactor enabling direct liquid contact between the cells and the liquid medium to be treated while at at the same time maintaining a homogeneous flow of liquid medium to all parts of said support.

It is another object of the invention to provide a method for the culturing of liver cells, with which liver cells can be kept viable in an amount and during a period of time that are practical for use in a bio-artificial liver.

A further objection of the invention is to provide a bio-artificial liver with improved therapeutical characteristics that can be used to replace and/or supplement the liver function of a patient.

Yet another object of the invention is to provide a method for the treatment of liver failure, especially acute liver failure, by using a bio-artificial liver.

Further objects of the invention will become clear from the description hereinbelow.

Brief description of the invention and the figures

It has now been found that an improved solid support for the cultivation of cells can be obtained by providing a 3D matrix material as described hereinabove, and in particular the matrix material according to GB-A-2,178,447, with hollow fibres for supplying and/or removing gaseous components such as oxygen and/or carbon dioxide, said solid support being especially suited for the cultivation of adherent tissue cells, such as human or animal liver cells.

It has also been found that an improved bio-artificial liver system can be provided using the solid support of the invention.

In general terms, the invention therefore relates to

- a solid support, comprising a 3D-matrix material and hollow fibres for gas transport;
- a method for preparing said solid support, comprising attaching hollow fibres to a 3D-matrix material;
- a biological reactor, comprising the solid support of the invention;
- a method for the cultivation and/or maintenance of cells, especially adherent tissue cells, and in particular liver cells, using the solid support and/or the bioreactor of the invention;
- a bioartificial liver system, comprising the solid support and/or the bioreactor of the invention;
- a method for replacing and/or supporting liver functions in a patient, and/or a method for the treatment of liver disorders, comprising the use of the bioartificial liver system of the invention.

Further aspects, embodiments and advantages of the invention will be made clear by means of the description hereinbelow and the figures, in which

Figure 1 shows a front view the preferred solid support of the invention;

Figure 2 shows a cross sectional view of the preferred solid support of the invention in the "sandwich" configuration;

Figures 3 and 4 show two possible geometries of the bioreactors of the invention;

Figures 5 to 9 show four possible configurations of the bio-artificial liver system of the invention;

Figure 10 shows an alternative embodiment of the solid support of the invention, comprising separate matrix sheets and hollow fibre sheets.

Figure 11 shows schematically an apparatus for immobilising cells within the bioreactor of the invention.

Figure 12 shows schematically a possible construction of a bioreactor of the invention.

Figure 13 shows a light microscopic photomicrograph of a cross-section of the 3D-matrix from a hepatocyte bioreactor cultured at 20.10^6 viable cells/ml for five days.

Figure 14 shows a scanning electron micrograph of isolated porcine hepatocytes cultured for five days in the 3D-matrix of the bioreactor device at 20.10^6 viable cells/ml.

Figures 15A and 15B show transactional flow sensitive MRI's of a small (A) internal diameter (1.32 cm) and a scaled-up bioreactor (B) internal diameter (2.2 cm).

The solid support.

The solid support of the invention in general comprises a 3D-matrix material and hollow fibres.

A 3D-matrix is defined herein as a material providing for a three dimensional for the growth of cells cultured therein. Such 3D-matrices are known to a man skilled in the art; Examples are:

1. Gelfoam (Gelatine, size: 20 mm*7mm, Upjohn Ltd., Tokyo Japan).
2. PVF (Collagen coated Reticulated Polyvinyl formal resin, size. 2 mm thick industrial filter material having a porosity of 80 %, Kanebo Kasei Co., Osaka, Japan).
3. PVLA-RPU (Poly-N-para-vinylbenzyl-lactonamide coated reticulated polyurethane, size: 34 mm diameter* 1 mm thick, Sanyo Chemical Industries, Ltd., Kyoto, Japan).
4. PGA (Polyglycolic acid), Albany International Research Co., Mansfield, Mass.
5. PVA (Polyvinylalcohol), Unipoint Industries, High-point, NC.
6. PGA/PLA (polyglycolic acid/polylactic acid, ratio 90:10), Ethisorb.

7. 3D-Polyurethane foam or non-woven matrix.
8. Porous siliconrubber foam (Ashby Scientific Ltd, Leicestershire, UK).

Preferably, said 3D-matrix is a material providing a high-surface area substrate, the effective surface of which is from 10 to about 100 times the area of the same projected onto a plane surface, comprising a physiologically acceptable network of fibres having a porosity from 40 to about 95% and a pore size of the order of 10 µm to 100 µm, or an open-pore foam structure with a pore size from about 10 µm to 100 µm, the overall height of the matrix being of the order of 50 µm to about 2000 µm, preferably 100-1000 µm, said matrix being in the form of a highly porous, non-woven sheet or mat.

This material, as well as its preparation, its advantages and its preferred embodiments, are described in the British Patent application 2 178 447, mentioned hereinabove and incorporated herein by reference.

The matrix preferably has an open pore foamed polymer structure with pores from about 10 µm to 100 µm and a porosity of from 60 to 95 %

The matrix can be made from any suitable material mentioned in British Patent application 2 178 447, but is preferably made from a polyester.

The matrix material can also be used in any form as described in British Patent application 2 178 447, but is preferably used in the form of a non-woven three dimensional fabric structure such as a sheet or mat; such flat, highly porous, non woven sheets or mats and their preparation are also described in said reference, and can be obtained commercially from Bibby Sterilin Stone, Staffordshire, U.K.

It is also possible to use a combination of several different 3D-matrix sheets, for instance a nonwoven polyester sheet and a nonwoven polyurethane sheet, or a combination of a nonwoven and a woven structure. It is also possible to use a 3D-sheet with a varying density,

i.e. a more open structure on the outside and a more compact structure on the inside, which can provide improved capture of cells during the loading of the bio-reactor.

When used in the preferred form of a sheet or mat, said sheet or mat preferably has a thickness of 10 to 1000 µm, more preferably 250 to 750 µm, and usually around 400-500 µm and comprises round, flat non-round or hollow fibres or a combination of such fibres of the order of from 0.5 µm to 20 µm in diameter or width, preferably 10 µm to 15 µm and/or preferred derniers of between 0.05 and 5 dpf as described in said reference.

Said fibres are preferably disposed in the sheet or mat as a highly disordered, random like, intertangled manner, the axes of the fibres forming an open multi-dimensional array.

For use in the cultivation of liver cells and/or in the BAL-system of the invention, the thickness of the sheet is preferably about equal 0.2 - 0.8 mm, preferably around 0.5 mm.

Although not critical, the sheet will generally have a width of 10 cm to 100 cm, usually around 20 cm.

The oxygenating hollow fibres used in the solid support should be permeable to at least gaseous oxygen and/or gaseous carbon dioxide, and as such both porous and non-porous ("closed") fibres can be used, with porous fibres being preferred. In other respects, the molecular weight cut off of the fibres is not particularly limited.

The fibres can be made of any suitable material, preferably a hydrophobic material, such as silicone, polyethylene, polypropylene, hydrophobic polysulfon, or any other suitable hydrophobic material from which hollow fibres can be made, or any combination thereof.

Such fibres and their preparation are known in the art; suitable commercially available materials are Silastic from Dow Corning (silicone fibres), Oxyphan and Plasma-phan from Akzo-Nobel (hydrophobic polypropylene fibres),

hydrophobic polysulfon fibres from Fresenius A.G., Bad Homburg, Germany, or polypropylene fibers coated on the inside and/or the outside with silicon rubber (Applied Membrane Technology, Minnetonka, Minnesota and Neomecs, St. Louis Park, Minnesota).

The hollow fibres can be treated with gas-plasma before incorporation into the matrix material so as to improve their hydrophobic properties.

The outer diameter of the fibres is preferably less than 10 mm, more preferably 0.05-5 mm, more preferably 0.1-1.0 mm.

The fibres are preferably evenly distributed throughout the matrix material. More preferably, they are aligned in a parallel fashion running from one end of the matrix material to the other end thereby providing ease of construction of the solid support, without the need of forming a complex network of intertwining hollow fibres.

The number of hollow fibres and the distance between the individual fibres in the solid support will be such that all cells adhering to the matrix material are sufficiently provided with oxygen and with sufficient removal of carbon dioxide.

In order to achieve this the distance between the individual fibres, measured from the centre of one fibre to the centre of the next, will usually be less than 10 mm, more preferably 0.1-5 mm, even more preferably 1-3 mm and most preferably around 2 mm, total number of fibres being related to the total length of the fibre sheet. Preferably, the solid support comprises at least three, more preferably at least ten hollow fibres.

Usually, the reactor will contain 50 to 50,000, preferably 500 to 5000 hollow fibres.

Preferably, the hollow fibres are attached and/or physically bonded to said 3D-matrix material, although the invention is not limited thereto, and alternative embodiments will be described hereinbelow.

The fibres can be attached to the matrix material by means of any suitable method that does not impede the oxygen/carbon dioxide transport through the fibre wall. As such the fibres can be weaved into the matrix material, glued onto the matrix material, sewn onto the matrix material, bonded thereon by means of ultrasound.

Examples of matrix materials suited in the practice of the present invention comprising hollow fibres attached to a nonwoven polyester sheet, comprise the commercial hollow fibre mats obtainable from AKZO-Nobel, (Wuppertal, Germany) and of Microgon (Laguna Hils, CA, USA).

In order to improve bonding and/or not to damage the matrix material, the matrix material can first be laminated with a suitable polyamide or silicone sheet or a coarse polypropylene mesh as described in GB-A-2,178,447, after which the fibres are bonded to said sheet or mesh by the methods described hereinabove.

When the matrix material is in the form of a sheet or mat, the hollow fibres can be attached to both sides of the matrix material, but are preferably only attached to one side of the matrix mat.

According to a preferred embodiment, the general geometry of which is shown in figure 1, the matrix material of the invention consists of a 3D-polyester matrix 1 according to GB-A-2 178 447, provided with parallel hydrophobic porous hollow fibres 2 with a diameter of about 0.7 mm, that are spaced at a distance of about 2 mm, weaved into the matrix material or bonded to one side thereof.

Such a solid support material provides ease of manufacture, and can advantageously be used in the "sandwich" configuration shown in figure 2, comprising a plurality of sheets 1, wherein each sheet is on both sides surrounded with the hollow fibres 2, and visa versa, with 3 being the intraluminar space (fibre lumen) and 4 being the extraluminar space.

In this sandwich configuration, besides providing for improved supply and removal of gasses, the fibres advantageously also acts as a spacer between the individual fibre sheets, and serve as a baffle means and/or channeling means so as to provide for an uniform flow and distribution of the liquid medium through the extraluminar space 4 to all parts of the solid support.

Furthermore, the fibres 2 provide physical support to the matrix sheets 3, which is especially important when the solid support is to be subjected to high shear, such as a liquid flow.

When the sandwich configuration is used, it is possible that the fibres from individual layer to individual layer are at an slight angle to each other, or even perpendicular from layer to layer.

According to another preferred embodiment shown in figure 10, the solid support comprises a seperate 3D-matrix sheet 26 and a separate fibre containing sheet 27, for instance obtained by weaving fibres into a sheet or bonding individual fibres together, and such sheets and their preparation are well known in the field of hollow fibre preparation.

In such a seperate fibre sheet, the fibres can be parallel in one direction, or the sheet can comprise two, three or more sets of parallel fibres wherein the sets of parallel fibres are perpendicular or at an angle to each other. Such a sheet can for instance be obtained by weaving the hollow fibres in such a way that the desired number of hollow fibre sets as well as the desired angle between these sets is obtained.

In such a sheet comprising different sets of hollow fibres, the fibres can also be made of different suitable materials as mentioned hereinabove, dependent upon the final use of said set of fibres. They can also have different diameters, so lang as they can be interwoven to form the desired hollow fibre sheet.

The fibre containing sheet can be laminated onto a sheet of the matrix material. It is also possible to attach the matrix material, for instance in the form of flakes, to such a hollow fibre sheet. All these embodiments are also preferably used in a sandwich configuration as shown in figure 10.

In all other respects, this embodiment comprises the same preferred aspects and advantages as mentioned hereinabove.

Finally, although the solid support of the invention generally does not require a pre-treatment step before use, such as the hollow fibre reactors of the prior art, it is comprised within the scope of the invention to treat the entire solid support, or only the hollow fibres or hollow fibre containing mat, with extracellular matrix materials, such as Matrigel, poly-N-para-vinylbenzyl-lactonamide or collagen based materials, in a manner known per se, in order to further improve cell adhesion. Also, the solid support can be provided with a sheet of an inpermeable material such as polyamide, polyfluorethylene or or silicone, for instance by laminating it onto the matrix sheet or rolling it up or stacking it with the solid support of the invention, thereby to some extent forming small compartiments within the solid support for maintaining a homogeneous cell distribution.

Further advantageous embodiments will be clear to a man skilled in the art and are comprised within the scope of the invention.

Geometry and construction of the bioreactor

In general the bioreactor of the invention comprises a suitable vessel, consisting of a wall enclosing a space, provided with the solid support of the invention.

The solid support is preferably in the form of a mat or sheet, more preferably in the "sandwich" configu-

ration shown in figure 2. There are two preferred ways of obtaining said reactor geometry.

According to the embodiment shown in figure 3, the solid support 5 is present in the reactor 6 in the form of a spirally rolled-up mat or sheet of the matrix material, with 7 being the wall of the reactor vessel. According to this embodiment the reactor will usually be a cylinder.

According to the embodiment shown in figure 4, the solid support 8 is present in the reactor 9 as stacked-up layers, with 10 being the wall of the reactor vessel. According to this embodiment, the reactor will usually have a box-like shape. Also, the individual solid support layers can be stacked at an angle, for instance at a right angles, giving the perpendicular hollow fibre configuration mentioned hereinabove.

The solid support/bioreactor of the invention can also comprise an alternating sheet of matrix material sheets and hollow fibre containing sheets as shown in figure 10, or hollow fibre sheets with the matrix material present in between the sheets, or bonded to the sheets, or comprise a laminate of a matrix material sheet and a hollow fibre sheet, as described hereinabove.

In all these reactor geometries, it is possible that there are incorporated into the support/reactor two, three or more seperate sets of (preferably) parallel hollow fibres, wherein each set of fibres is at an angle or perpendicular to one or more of the other sets of fibres present in the reactor.

These different sets of fibres can be obtained by any of the methods described hereinbove, for instance by stacking individual layers of matrix material with hollow fibres physically attached thereto at an angle to each other, by using seperate hollow fibre sheets comprising two, three or more individual sets of fibres at (an) angle(s) to each other as described hereinabove, by using seperate sheets of matrix material and hollow fibre

material and placing the hollow fibre sheets at (an) angle(s) to each other, or any combination thereof, such as the use of a sheet of matrix material with hollow fibres physically attached thereto stacked at an angle with a seperate hollow fibre sheet.

When there are several sets of hollow fibres, these sets can be made from the same of different suitable hollow fibre materials and can have different diameters etc., dependent upon the final use of said fibre set.

Further suitable reactor geometries will be clear to a man skilled in the art, and will be comprised within the scope of the invention. In any case the geometry will be such that during use all the cells in the bioreactor are in suitable proximity to the oxygenation fibres, so that they can be provided adequately with oxygen.

Also, for most applications, and especially for use as or in a BAL, the reactor geometry is preferably such that most or preferably all of the liquid medium perfused through the reactor vessel will come into contact with the cells immobilised on the solid support.

All the above mentioned reactor geometries comprising two or more sets of hollow fibres should however be distinguished from the reactor geometry described by Gerlach et al comprising a three dimensional network of hollow fibres: In the Gerlach reactor, there is no seperate 3D matrix material for cell adhesion, so that the cells have to adhere to the hollow fibres themselves. For this to be possible, the hollow fibres used in the Gerlach reactor have to be interwoven as such to form the required three dimensional network. Also, in this network, the distance between the individual interwoven hollow fibres must be so small so as to make three dimensional adhesion of the liver cells to these fibres possible. It will be clear that this will make the 3D hollow fibre network according to Gerlach et al very difficult and expensive to produce.

According to the present invention, cell adhesion is essentially provided by the 3D matrix material, and not by the hollow fibres, so that a three dimensional hollow fibre network is not required for providing the 3D cell attachment. (For instance, in all the above reactor geometries, there is an essentially two dimensional hollow fibre network with the sets of fibres lying in the same -in the case of interwoven fibre sets- or in parallel -in the case of stacked fibre sets- plane(s) within the solid support).

It will be clear that because of this the distance between the individual hollow fibres in the solid support the invention is less critical and can be larger than in the Gerlach reactor, as long as sufficient oxygenation of all the cells present within the matrix can be obtained.

Also, the solid support of the invention with different sets of fibres is easier to manufacture simply by stacking of by using a two dimensional fibre sheet comprising two or more sets of parallel fibres.

The reactor of the invention will usually comprise a gas inlet/outlet operably connected to the hollow fibres, so that gas can be fed to and removed from the hollow fibre lumen.

The reactor will usually also comprise at least one liquid inlet and outlet, operably connected to the extra fibre space, through which a liquid medium can be fed to or removed from said extra fibre space or the cells present therein. The reactor can comprise additional inlets and outlets for both gasses, liquids and/or solids, as required.

The reactor can further comprise all known elements of biological reactors, such as gas and/or liquid pumps operably connected to the different inlets or outlets; means for measuring and/or controlling the temperature within the reactor vessel; access means, such as a hatch, for accessing the inside of the reactor; inspection means; probes and means for inserting them, such as

probes for the measurements of the viability as further described hereinbelow, etc.

The reactor may further be provided with means for the automatic control of the different reactor functions, such as a computer means operably connected with the pumps, temperature controlling means etc.

The reactor may also be provided with means for agitating the reactor, such as an electric motor, for instance for rotating the reactor along one of its axes, or with means for stirring inside the reactor, although the latter is usually not preferred.

The wall of the reactor vessel can be made of any suitable inert material, such as glass, plastics such as plexiglass, or metals, and polycarbonate or polysulfon, the latter materials being suited for the preferred steam sterilization. The inside of the reactor vessel can be provided with a special coating compatible with the cells to be cultured.

The size of the reactor is not limited and will usually depend upon the capacity required. The volume of the reactor can therefore vary from 1 ml to 1000 liter.

It will be clear, that for the above reactor geometries, the solid support of the invention, especially when used in the form of a sheet or mat with the hollow fibres attached to it, provides for ease of construction, especially compared to the capillary network containing bioreactor of Gerlach et al as described hereinabove. The solid support can also advantageously be used for adapting an existing reactor for use with the method of the invention.

For instance, the reactor geometry of figure 3 can simply be obtained by rolling up a sheet of the solid support of the invention and bringing said rolled up support sheet into the reactor vessel. It will be clear that the size of the solid support will be such that once rolled up, it will fit into, and preferably have a size essentially corresponding to, the size of the reactor

vessel. If necessary the solid support can be cut to the desired size either before or after it has been rolled up. Similarly, the reactor geometry of figure 3 can be obtained by stacking up one or more sheets of the solid support of a suitable size, or folding one or more sheets into the reactor vessel.

As mentioned hereinabove, it is also possible to roll up a separate sheet of matrix material and a fibre containing sheet, or to stack up separate alternating layers of matrix material and fibre containing material.

In all these configurations, the hollow fibres will provide physical support to the rolled up or stacked up solid support, as well as provide for improved liquid flow through the extra fibre space.

It will be clear that in general, the amount of solid support present inside the reactor vessel as well as the dimensions thereof will usually be dependant upon and/or adapted to the volume and the dimensions of the reactor vessel.

The reactor vessel can also contain means for supporting and/or keeping in place the rolled up or stacked up solid support, as will be clear to a man skilled in the art.

After the solid support has been put into place inside the reactor vessel, both the hollow fibres and the extra fibre space can be operably connected to the various gas inlets and outlets and fluid inlets and outlets, respectively, that will usually form part of the reactor vessel, optionally through or by means of distribution means that can provide for an even distribution of the gas flow and/or the liquid flow through the reactor.

According to the preferred embodiment of the invention, wherein the fibres are unidirectional, the fibres are on one side of the matrix material collectively connected to an gas inlet supply and on the opposing side collectively connected to a gas outlet.

However, it is also possible to have a system working in countercurrent, i.e. where the direction of the gas flow is opposite from fibre (layer) to fibre (layer), or at right angles with the perpendicular configuration mentioned hereinabove.

It will be clear from the above that the reactor according to the present invention is also much easier to operate than reactor of Gerlach et al comprising a woven network of four discrete capillary membrane systems, which therefore requires multiple inlet and outlet systems. Also, compared to the reactor of Gerlach et al, the solid support of the invention comprising a 3D-matrix material provides both improved cell attachment and improved cell capacity per unit volume.

According to the invention, the extracellular fibre space can be connected directly to a liquid inlet/outlet system, which makes it possible to perfund said extra fibre space with liquid medium, giving direct liquid contact between the cells present in the extra fibre space and the liquid medium.

Although the invention in it simplest embodiment only comprises one set of hollow fibres for the supply and/or removal of gaseous components, it is to be understood that further separate hollow fibres systems for the supply and/or removal of specific gasses and/or liquid media can be provided. These further systems can also be used for the separate controlled introduction of gaseous, liquid and/or dissolved components independent from the main gaseous or liquid feed as described hereinabove. It is also possible to use individual fibres, sets of fibres or fibre layers of the solid support for this purpose, as long as sufficient oxygenation can be maintained.

For such applications, the solid support/bioreactor of the invention will usually comprise two or more different sets of hollow fibres parallel of at (an) angle(s) to each other as described hereinabove, with

each set of fibres being used for a specific purpose, with at least one set being used for sufficiently oxygenating the reactor according to the invention.

It is also possible according to the invention that different sets of hollow fibres are used for inflow and outflow. In this embodiment, the fibre will usually be closed at one end and at the other end be connected to an inlet or outlet respectively, said inlet and/or said outlet optionally being provided with pumping means.

The gaseous or liquid medium is supplied through the inlet to the fibre lumen of the inlet fibre, passes through the fibre wall into the extra fibre space and is then taken up by the outlet fibre and removed.

Although there may be problems with regard to the achievable flow and/or ths fibres getting clogged, the main advantage of this embodiment will that all the medium supplied will necessarily come into contact with the cells in the extra fibre space.

Further advantageous embodiments will be clear to a man skilled in the art and are comprised within the scope of the invention.

Cultivation of cells in the bioreactor

The bioreactor of the invention can be used to culture and/or maintain all kinds of cells, and the invention further relates to such uses and methods of cell cultivation and/or maintenance.

Preferably, the cells are plant, human or animal derived adherent cells, such as tissue cells, although fungal cells, as well as all kinds of one-cell organisms such as bacteria can also be cultivated with advantage.

The invention can also be used for the cultivation of modified cells such as cell lines, fused cells, hybridomas, transformants etc.. Further examples of suitable cells will be clear to a man skilled in the art.

The solid support and reactor of the invention can

also be used for cultivating two or more different types of cells at the same time.

In general, the invention is especially suited for the cultivation of cells that put stringent requirements on the solid support available for cell attachment, the supply and/or removal of gaseous components, such as oxygen, or both. The advantageous properties of the solid support of the invention further make it possible to culture and/or maintain cells at very high cell densities and with excellent "three dimensional" cell attachment and cell proliferation, as mentioned hereinabove.

The total cell capacity of the reactor will usually be dependant upon factors like the size of the reactor, the amount of solid support present therein and the type of cells used.

In general, because of the very large surface area available for cell attachment, the improved oxygenation and the attainable high cell densities, the reactor will usually show a high cell capacity per unit volume. Also, because of the improved oxygenation and the homogeneous liquid flow, the reactor can be scaled up to the required capacity - for instance by increasing the volume and/or the amount of solid support present within the reactor in a manner known per se - without the problems of scale usually associated with large bioreactors, such as insufficient oxygenation and/or inhomogeneous liquid flow.

The reactor of the invention is especially suited for the cultivation of human liver cells or animal liver cells, such as dog or pig liver cells, both as primary cells or as immortalized cells.

The reactor can further be used for the cultivation and maintenance of liver-cell derived cell lines, liver cell transformants; and hepatoma cells and hepatoblasts, as well as cell lines derived therefrom, such as the transformed C3A-hepatoma derived cell line described by Sussman et al.,incorporated herein by reference.

The term liver cells and/or the equivalent term hepatocytes as used in the present application therefore comprises all these different types of cells and cell lines.

Methods for obtaining said cells, such as isolation, culturing, transformation, etc, are well known and are for instance described in the abovementioned prior art, incorporated herein by reference.

Although for use in a BAL, preferably liver cells with a viability of more than 80% are used, for reasons mentioned hereinbelow, the BAL-system of the invention also makes it possible to use liver cells with a viability of no more than 70 %, or even as low as 40-50 %.

This means that the present invention puts less stringent requirements on the cell viability than the prior art BAL-systems that require a viability of more than 80 %. This is an important practical advantage in view of the problems normally associated with attaining such a high cell viabilities, especially with primary liver cells, as described hereinabove.

Furthermore, the invention makes it possible to use liver cells that have been stored by means of cryopreservation, which usually affords liver cells with a viability of less than 60-80 %, so that the isolation of fresh liver cells with sufficient viability for each new BAL is no longer required. Again, this was not possible with the prior art BAL-systems.

If desired, the culture and maintenance of the liver cells can be carried out in the presence of added supplements such as growth factors, antibiotics and hormones, as well as added attachment factors and extracellular matrix constituents. These can be added to the perfusion flow itself before it enters the reactor of by means of seperate means provided in the reactor, such as a seperate set of hollow fibres provided for this specific purpose.

Also, the invention can be used for the co-culture of liver cells, for example with non-parenchymal liver cells. Optionally, this can be carried out in seperate hollow fibres present in the reactor.

Although these techniques are known in the art, vide for instance the abovementioned references, due to the advantageous properties of the solid support of the invention, their use is not always necessary and certainly not required.

The bioreactor can also be used for culturing hybridoma cells -i.e. for the production of monoclonal antibodies- which usually show poor attachment and/or adherence to solid supports.

For cultivating the cells, the cells are in general introduced into the bio-artificial reactor system, after which they are allowed to attach and/or adhere to the solid support during a suitable period of time. During this attachment phase, an oxygen-containing gas or gas mixture through the hollow fibres, such as pure oxygen, air, or a gas mixture containing oxygen, preferably 50-99% oxygen, more preferably 90-99% oxygen, in admixture with another inert and/or physiologically acceptable gas such as nitrogen or carbon dioxide is led through the oxygenating hollow fibres and spent gas is removed via the gas outlet.

Transport of these gasses to the cells will essentially take place through diffusion, whereby sufficient gas exchange is assured both by the high available surface area of the hollow fibres as well as the small distance between the cells and the nearest oxygenating fibre. This diffusion-oxygenation avoids the limitations associated with oxygenation by the liquid medium, as well as the use of a separate oxygenator, because the oxygenator is directly incorporated into the reactor itself.

Also, nutrients can be fed to the attached cells, and waste products can be removed, in general by means of

a extra luminal liquid flow. As such, all known and suitable nutrients and nutrient containing solutions and media can be used, or the solution can be especially adapted to the needs of the cells to be cultured.

Said nutrient medium is preferably fed from one side of the matrix material and removed from the other side -i.e. by means of an unidirectional flow- together with formed by-products and waste products.

It is also possible to provide separate fibres for the controlled feeding of some specific nutrients, although this will in general involve a more complicated construction and operation of the reactor, which for that reason is not preferred.

During cultivation, the cells can be kept at a desired, biologically or physiologically acceptable temperature, i.e. by keeping the reactor in a thermostat, or by controlling the temperature of the extra luminal liquid flow and/or the gas flow within the fibres, as will be clear to a man skilled in the art.

In cultivating the cells, the reactor can be loaded with a small amount of cells, after which the cells are allowed to divide so as to fully populate the reactor. According to this embodiment, the solid support of the invention will require less heavy inoculation charges than prior art supports, with inoculations with amounts of 10% or less or even as low as 5% of the total cell capacity being sufficient so as to fully populate the reactor by advantageous "three dimensional" growth.

It is also possible to feed more cells into the reactor, so as to fully saturate the matrix material with adherent cells, or even to use an excess amount of cells, after which superfluous cells are removed.

Whether loaded with a small amount of inoculum, or with a large excess, the 3D-matrix support of the invention will provide for increased "capture" of the cells, so that cells with sub-optimal attachment can be used and/or the time needed for the attachment phase is con-

siderably shortened. The latter is of special advantage when the reactor is to be used as a BAL, because the time needed until a BAL is ready for use is critical in a clinical setting.

Also, because of the extra-luminal channels formed within the solid support by the hollow fibres acting as a spacer means, channeling means or baffle means, after introduction into the bioreactor, for instance by injection of a cell suspension, the cells will be distributed quicker and more evenly over the entire support, reducing the time needed for the attachment phase even further and resulting in a more homogeneous cell distribution.

In general, the attachment phase will take from 30 minutes to 5 hours, dependant upon the specific cells used.

According to one special aspect of the invention, if the cell sample to be introduced in the reactor contains both viable and non-viable cells, the unique design of the reactor makes it possible to separate viable from non-viable cells, as will be described hereinbelow with reference to the cultivation of liver cells.

Further advantages of the bioreactor of the invention is that during the attachment phase the sedimentation of the cells as a large pellet on the bottom of the bioreactor can be precluded. This will also be further described hereinbelow.

After the attachment phase, and optionally a cell growth phase and/or the attainment of a steady state, the seeded reactor will generally be ready for its intended use. During such use, the cells will usually be maintained in/at sufficient quantity, viability and activity, i.e. by maintaining biologically and/or fysiologically acceptable conditions, while the liquid medium to be treated is fed to the cells, usually through the extra fibre space. For most uses and with most cells, the bioreactor will make it possible to maintain viability and

activity at higher levels during a longer period of time than prior art methods.

Although the invention will be further described hereinbelow with respect to the cultivation of liver cells and the use as a bio-artificial liver, it is expected that the bioreactor of the invention can also advantageously be used for other bio-artificial systems.

As such, the solid support and/or bioreactor of the invention can for instance be used in a bio-artificial pancreas, a bioartificial kidney and/or a bioartificial parathyroid gland, artificial bone marrow, systems that are currently based on hollow fibre reactors as described hereinabove. Use of the invention in these systems will also result in the advantages of the invention, such as improved cell attachment and capacity, direct contact of the cells with the liquid medium and/or improved oxygenation, as well as longer effective working time.

The bioreactor of the invention can also be used for the production, bioconversion and/or removal of substances in or from a liquid or gaseous medium, using cells capable of the desired biological reactions. For these and other applications, the invention advantageously provides for a high surface area available for gas exchange between the fibre lumen and the extra-luminal space through the hollow fibre wall, as well as for direct liquid contact between the cells within the extracellular space and the liquid medium, the latter being of particular importance in the degradation, bioconversion or production of high molecular weight biological substances such as polypeptides. The produced substances can than be isolated from the liquid medium derived from the bioreactor in a manner known per se.

Other advantageous uses of the bioreactor of the invention will be clear to a man skilled in the art.

Use of the solid support and the bio-reactor of the invention as a bio-artificial liver.

As stated hereinabove, the advantageous properties of the solid support and the bioreactor of the invention make them especially suited for use in or as a bio-artificial liver system.

In such a system, suitable liver cells are cultured and/or maintained using the solid support and/or the bioreactor of the invention as described hereinabove.

Therefore, in general, the bio-artificial liver system of the invention comprises a bioreactor of the invention and will as a rule also comprise liver cells as defined hereinabove, which usually will be present in the extraluminar space, more particularly be attached to the matrix material of the solid support.

During use, the bioreactor is operably connected to the blood circulation of a patient by means of a liquid circuit, so that a liquid medium directly or indirectly derived from the patient is fed to the liver cells in the extra luminar space, after which said cells are allowed to carry out most or all of the functions normally carried out by the liver in vivo. After treatment by the liver cells, the liquid medium is returned to the patient.

The BAL-system of the invention will therefore further comprise a liquid circuit for circulating the liquid medium, as well as pumping means known per se for controlling the liquid flow through said circuit. As such, the bioreactor of the invention can be incorporated into any such circuit known per se, for example as described in the abovementioned prior art, incorporated herein by reference, in which the bioreactor of the invention will replace -for instance- the hollow fibre BAL-system.

The circuit can also contain further means for the treatment of the liquid medium, such as an activated charcoal column for the absorption of hydrophilic toxins and/or a resin column for adsorbtion of hydrophobic substances (e.g. bilirubine).

The liquid circuit may also comprise cell filters for removing cells from the liquid flow. When used to keep dead liver cells away from the patients circulation, this cell filter will usually be placed after the bioreactor.

The liquid circuit may also comprise means for adding nutrients and other desired substances to the liquid medium, although in this respect the liquid medium derived from the patient may itself be sufficient for keeping the liver cells in the reactor viable. Also, separate fibre systems for adding nutrients may be provided in the reactor as described hereinabove.

During use, the BAL-system of the invention can be perfused with whole blood - either arterial or venereal - derived from a patient in a manner known per se. In this case the liver cells in the reactor need to be immunologically compatible with the patients blood, so that usually human liver cells or cells and cell lines derived therefrom will be used. The less preferred use of xenocytes could require the use of immunosuppression.

However, the BAL of the invention is preferably perfused with plasma derived from a patient. In this preferred mode of plasma perfusion, the circuit will usually comprise a plasma separator or plasmapheresis unit for separating the plasma from the whole blood derived from the patient. The use of BAL-systems on the basis of plasmapheresis, as well as suitable plasmapheresis units, are well known in the field and are for instance described in the above prior art, incorporated herein by reference.

In its plasmapheresis mode, the circuit may also comprise immunological barriers for keeping the patients blood circulation immunologically separate from the plasma circulation through the reactor, making it possible to use xenocytes such as pig hepatocytes without the need of immunosuppression. Usually, the plasma separator/ plasmapheresis unit itself will provide to some extent

for said immunological separation. The circuit can however also contain (further) separate means, such as membranes, columns or hollow fibre modules with a suitable molecular weight cut off, as described hereinabove, either placed before or after the reactor, and/or specific columns for the adsorbtion of antigens and/or antibodies etc. such as are known to a man skilled in the art.

As mentioned hereinabove, a separate oxygenating unit does not have to be incorporated into the liquid medium circuit, even when plasma-perfusion is used.

A number of possible configurations of the BAL of the invention in the preferred plasmapheresis mode are shown in figures 5-9.

Figure 5 shows a configuration in which arterial blood from the patient is fed through line 11, optionally by means of pump 12, to the plasmapheresis unit 13, where the plasma is separated from the whole blood, which is led back to the patient through line 14.

The plasma is then fed directly through line 15, optionally by means of pump 16 to the liver cells containing bioreactor 17, and from there returned to the directly venous blood in line 14 by means of line 18.

An oxygen containing gas is fed to the hollow fibres by means of feed 19, and the carbondioxide enriched gas is led away through line 20.

Figure 6 shows a "high flow loop" configuration designed for recirculation of the plasma over the reactor by means of additional line 21 provided with pump 22.

In figure 7, the circuit is provided with a cell filter 23 for keeping dead cells flushed out of the reactor away form the patients circulation.

In figure 8, the circuit is provided with a hollow fibre membrane cartridge 24 for immunological separation placed in the high flow loop after the reactor.

In figures 9a and 9b the circuit is provided with a immunological pretreatment column and/or columns for

hydrophilic and/or hydrophobic toxin removal, as described hereinabove step 25 either incorporated into (fig.9a) or outside (fig.9b) the high flow loop.

It will be clear to a man skilled in the art that all the different equipment mentioned above and shown in the figures can be combined into one circuit.

The different elements of the BAL-circuit may be provided as an integrated system in a single housing, or the BAL may consist of separate connected elements.

Although dependant upon the geometry and capacity, the amount and activity of the cells present in the reactor, the desired therapeutical application and other such factors, the BAL of the invention can be used to treat 1 to 300 ml of liquid medium derived from a patient per minute.

In order to achieve this, the liquid medium can be fed directly to the reactor 17 in at a corresponding rate, as shown in figure 5.

However, preferably the bioreactor of the invention is incorporated into a "high flow loop", as known per se from the abovementioned prior art, and shown in figure 6.

In such a loop, formed by reactor 17, line 21 and pump 22, and part of lines 15 and 18, as shown in figure 6, the flow of the liquid medium over the reactor 17 can be kept at a higher rate than the flow of liquid from the patient through lines 11 and 14, thereby providing for recirculation of the liquid medium over the reactor 17. Usually, this will be carried out by suitable control of pumps 16, 22 and 18a, by keeping them at a suitable flow ratio, usually from 1:2:1 to 1:100:1, respectively.

The BAL of the invention can also comprise two or more bioreactors of the invention connected in series and/or in parallel; for instance containing the same type and/or different types of liver or other cells.

The bioartificial liver system of the invention can be used to support and/or replace liver function in patients with impaired liver function and/or in cases in

which artificial liver support is desirable and/or required. The BAL-system can for instance be used in patients suffering from fulminant hepatic failure (FHF), for instance due to viral hepatitis infections or acute liver poisoning (for instance with acetaminophen, CCl_4, alcohol or drugs), as well as transient liver ischaemia, and liver trauma due to injury. The artificial liver can also be used to improve the patient's condition before liver transplant, to bridge the period before liver transplant, to bridge the rejection period after acute rejection of a transplanted liver, during the anhepatic phase while a liver transplant is carried out and/or during recovery of a liver transplant, or to allow time to regenerate the patient's own liver.

Furthermore, the BAL system of the invention can be used in the treatment of chronic liver diseases to enhance the quality of life of the patient and/or to bridge periods of exacerbation.

The BAL-system of the invention can also be used to bridge patients through a relatively brief crisis period allowing their own livers to regenerate thereby sparing the trauma and expense of transplants.

As such, the BAL of the invention will preferably be used continuously, although intermittent use is also envisaged.

In order to obtain the bioreactor, the liver cells can be introduced into the bioreactor in a manner known per se and/or as described hereinabove.

According to one embodiment, only a small number of cells is seeded into the reactor, after which said cells are allowed to grow and divide, until the bioreactor has reached its maximum capacity and/or a steady state is reached after which the reactor can be used as a BAL.

In this embodiment, the support and bioreactor are therefore used for cultivation of the liver cells as well as the bio-artificial liver system itself. It will be clear to that according to this aspect of the invention,

the 3D-solid support of the invention will favour cell growth and cell division, especially compared to the hollow fibre systems of the prior art, in which cell growth and division will usually be limited or even precluded.

This embodiment will usually not be suited for liver cells that are unable to divide and grow after they have been isolated, such as primary liver cells. It is however expected that even with primary liver cells, the solid support of the invention will favour some growth and division to occur, especially compared to the prior art. The cultivation of primary liver cells using the solid support and/or the bioreactor of the invention, optionally with the use of growth factors etc., is therefore expressly included within the scope of the present invention.

According to another embodiment, the amount of cells will largely correspond to and/or exceed the capacity of the bioreactor. In this case the cells are added to the reactor and allowed to adhere to the solid support, after which the excess non-attached cells are removed, for instance by washing. After that, the bioreactor can be used as or in a BAL, optionally after attainment of a steady state.

This embodiment will generally have the advantage that the BAL-system is ready for use after a shorter period of time compared to prior art systems, according to the invention usually between 0.5 to 6 hours, in most cases around 2 hours. Also, this embodiment is especially suited for use with primary liver cells.

In general, the BAL will be seeded with 1.10^5 - 1.10^8 cells/ml, usually around $1\text{-}50.10^6$ cells/ml (unit volume), and to a total capacity of 10^8 to 10^{11} cells, i.e. around 1-1000 g cells, preferably 100-500 g cells.

The reactor is usually seeded by injecting a suspension of the liver cells into the reactor, obtained for instance by cultivation of the cells, by suspending liver cells in a suitable liquid medium, or after isolation,

after which the cells are allowed to distribute themselves throughout the reactor and adhere themselves to the solid support during a suitable period of time, usually from 1 minutes to 5 hours or more, preferably about 30 minutes to 3 hours, and more preferably around 2 hours. For comparison, with the reactor of Gerlach et al, the attachment phase can take up to 8 hours or more.

In order to facilitate the distribution of the cell suspension even further, the reactor can be agitated after the cell suspension has been injected.

According to a highly preferred embodiment, after injection of the cell suspension, the reactor is rotated, intermittently but preferably continuously, around its longitudinal axis, i.e. the direction of the hollow fibres in the solid support, during the abovementioned period of time at a speed of 0.01 - 100 rpm, preferably 0.1 - 10 rpm, more preferably around 1 rpm. In the preferred embodiment shown in Figure 11 the bioreactor 28 shown in Figures 3/4 is rotated around a central axis 29, the reactor being attached to the axis by means of attachment means 30, such as a clamp of suitable size (not shown).

This method of distributing the cells throughout the reactor prevents the formation of a cell pellet at the bottom in the bioreactor, which would lead to mass transfer problems during use, especially with regard to the cells at the centre of the pellet, which can lead to loss of functional activity and/or viability. By rotating the reactor, and preferably periodically inversing the direction of rotation, the direction of sedimentation continuously changes, and the cells in suspension can be considered to follow an almost circular path through the reactor, so that the cells repeatedly come into contact with the matrix material, thereby greatly enhancing the chances of entrapment by the polyester fibres in said matrix material, so that a homogeneous immobilisation at a high rate and speed of attachment is obtained.

After immobilisation of the cells is complete, the remaining suspension containing non-adhered and/or excess cells is removed from the reactor, after which the reactor can optionally be flushed and/or with a suitable liquid medium.

Also, by agitating, and preferably rotating the reactor, living cells can be separated to at least some extend from dead cells present within the injected cell suspension, especially when a cultured suspension of primary liver cells is used. The living cells are entrapped by and/or adhere to the solid support, whereas the non-adhering dead cells are removed with the remaining suspension or by washing the reactor.

Also, by perfunding the reactor with a suitable liquid medium, dead cells can be flushed out of the reactor. This "flushing out" of dead cells can even take place during use, i.e. while the reactor is connected to the perfusion circuit. In this case, incorporating a cell filter 23 into the liquid circuit after the reactor, as shown in figure 7, will be highly advantageous.

This favorable removal of dead cells cannot be achieved with the prior art hollow fibre bioreactors, because in these reactors, the liver cells essentially are present in an enclosed "compartiment" between the hollow fibres, without means for perfunding said space, or captured within a (hydro)gel.

During use, the liver cells in the reactor are maintained in a manner known per se. The BAL of the invention is preferably kept at a physiologically acceptable temperature, preferably around 37 C. Also, additional nutrients and other suitable substances may be added to the reactor, as and if required.

The liver cells are oxygenated by feeding an oxygen or an oxygen enriched gas such as "carbogen" (a 95:5 O_2/CO_2 mixture) or a CO_2-enriched oxygen containing gas, such as "culture gas" (95 % air, 5 % CO_2). This gas can be fed by any suitable means, such a gas-cylinder or a

gas pump, or by connection to an external gas supply. As stated hereinabove, this method of direct oxygenation through closely packed hollow fibres means that the generation of deleterious oxygen and/or carbon dioxide gradients is avoided. Also, during perfusion, the liquid medium used can have a further "mixing" action on the gas supply, reducing said gradients even further.

Before or during use, the functional effect and the metabolic performance of the bioreactor and the cells contained therein can be monitored in a manner known per se with any of the large number of tests available for this purpose, such as measurement of protein synthesis, ureagenesis, oxygen uptake (for which advantageously direct measurement at the gas inlet and gas outlet can be used), cytochrome P450-activity, drug metabolic assays, clearance techniques etc, vide for instance Rozga et al, mentioned hereinabove and incorporated herein by reference. Also, a biomass meter (Aber) can be used, which uses conductivity measurements based upon differences in membrane potential between dead cells and living cells. Such a meter is known to a man skilled in the art.

The use of the bio-artificial liver of the invention will of course afford all the advantages associated with the use of the solid support and the bioreactor of the invention, as well as a number of further advantages, such as:

- Improved attachment of the liver cells and improved cell capacity per unit volume due to the presence of a suitable matrix material. The solid support also offers an improved environment for cell growth and cell division.
- Improved oxygenation of the liver cells due to the presence of the oxygenation fibres, without the need of a separate oxygenator and without the occurrence of deleterious gradients.

- Direct liquid contact between the liver cells and the blood or plasma to be treated, without the need for toxins and/or liver secretions to pass a membrane with the associated mass transfer and molecular cut off problems.
- The reactor can easily be "scaled up" to the capacity required for therapeutical use.
- A simple construction that can easily be manufactured and operated, in its simplest form requiring only one fluid inlet/outlet and one gas inlet/outlet. Also, no expensive pre-treatment of the solid support is required.
- Compared to the prior art systems, the bioreactor of the invention puts less stringent requirements on the (primary) liver cell preparations used, especially with regard to viability and attachment.
- The speed and rate of cell attachment after seeding is reduced, so that the time until the BAL is ready for use is shortened and less liver cells are required.

Finally, a major advantage of the use of the 3D-solid support and the bioreactor of the invention as a BAL and/or in the cultivation and/or maintenance of liver cells, as well as the abovementioned rotation method for seeding the reactor, is that the cells will be present and/or maintained in the reactor as small cell aggregates, in at least one diameter not being larger than 10 cells, preferably being not larger than 6-8 cells (100 µm). It is well known in the art that such hepatocyte aggregrates function and remain viable during a longer period of time, are more active and better differentiated than hepatocytes grown in monolayers or on 2D-carriers or hollow fibres. Also, the morphology of the cells cultured in such small aggregates is similar to the morphology of liver cells in the liver in vivo. Also, as these aggregates are of relative small size (only 6-8 cells in diameter) there are no problems with regard to mass transfer to the cells at the center of the aggregates, as with liver cells cultured in large (> than 200 µm) aggregates,

such as the 500 µm aggregates in the reactor by Gerlach et al., or immobilised in microcapsules.

The invention therefore makes it possible to cultivate and/or liver cells in a high capacity reactor at very high cell densities, i.e. 20-40 x 10^6 cells/ml or more. It can also be said that, in general and compared to the BAL-systems of the prior art, the solid support of the invention provides an environment that more closely matches the biological conditions/environment of the cells in the liver.

Of course, these charactistics also mean that the BAL of the invention has great advantages from a therapeutical point of view, especially compared to the prior art systems. The BAL will generally be therapeutically effective for a longer period of time, show improved efficiency and can be easily provided with sufficient capacity for liver replacement.

A further practical advantage of the BAL of the invention is that it can be sterilized in an autoclave (20 minutes at 120 C). Prior art systems require gas sterilisation with toxic gases such as ethylene oxide, which is still present in and given off by the reactor fibres weeks after the reactor has been sterilized.

Finally, the BAL of the invention for the first time makes it possible to succesfully employ cryopreserved primary hepatocytes in a bioartificial liver system, opening the possibility of centralized isolation and preservation, after which the cells can be distributed to the hospitals where they can be stored until they are needed. Together with the shortened attachment phase of the BAL of the invention, this means that in a clinical setting, the BAL of the invention can be put at the disposal of physician sooner and at lower costs.

The invention will now be illustrated by means of the following non-limiting examples.

EXAMPLE I: in vitro tests

The development of a liver support system for the treatment of patients with fulminant hepatic failure and as a bridge to liver transplantation is a significant challenge. Many early attempts focussed on blood detoxification based on the assumption that liver failure could be reversed if the associated toxins were removed from the circulation of the patient. Although improvement of the neurologic status in patients has been reported, none achieved long-term survival. It was therefore concluded that an effective liver support system should be able to perform the liver's multiple synthetic and metabolic functions, including detoxification and excretion. The most logic approach to this problem is the introduction of active functioning hepatocytes. The state-of-the-art embodiment of this theory is presented in the bioartificial liver (BAL), an extracorporeal device comprising well nourished and oxygenated viable hepatocytes immobilized on a mechanical support and separated from the blood circulation by semipermeable membranes.

Objectives like biocompatibility, maintenance of functional capacity and practicality, important aspects in the development of the BAL, have been discussed in the prior art. However, the current bioreactor designs do not meet the essential conditions for optimal mass transfer to and from the hepatocytes as present in the intact liver. In this respect, the impact of the bioreactor construction on hepatocyte function has been undervalued.

The aim of our study was to develop a bioreactor configuration that allows high density hepatocyte culture and simultaneously ensures every hepatocyte to operate under in vivo like perfusion conditions and direct medium contact, thereby more closely mimicking physiological mass transfer. In addition, we wanted to culture hepatocytes as small aggregates, known to maintain many of the cyto-architecture characteristics found in vivo and

exhibit higher and prolonged functional activity compared with hepatocytes cultured as monolayers.

Another goal was to develop a bioreactor which can be scaled up to incorporate sufficient cell mass for therapeutic liver support. This resulted in a novel bioreactor design comprising a spiral wound 3D nonwoven polyester matrix and an integrated oxygenator in which hepatocytes reorganize and immobilize as small aggregates.

Hereinbelow, the characteristics and the in vitro results of the novel bioreactor design of one embodiment of the invention are presented.

1. Materials and methods

1.a Hepatocyte isolation

Pig livers were kindly provided by the department of clinical and experimental cardiology of the AMC, Amsterdam, the department of dermatology of the AMC, Amsterdam, and a local slaughterhouse. The hepatocytes were isolated from pigs with a body mass of 20-25 kg using a simple two step collagenase perfusion technique as described previously (te Velde AA, Ladiges NCJJ, Flendrig LM, Chamuleau RAFM, J Hepatology 1995; 23: 184-192, incorporated herein by reference.). The viability of the isolated cells based on trypan blue exclusion varied from 71 to 96% (n=8, mean 89±7%). The yield varied from 8.10^6 to 30.10^6 hepatocytes per g wet liver weight for the different isolations.

1.b Bioreactor

The bioreactor is based on a 3D nonwoven polyester fabric especially designed for culturing anchorage dependent cells (Bibby Sterilin Ltd, Stone, Staffordshire, GB) and hydrofobic polypropylene hollow-fibres donated by Dr. J. Vienken of AKZO-NOBEL (Plasmaphan, AKZO-NOBEL, Wuppertal,

Germany) for oxygenation and carbon dioxide removal. The 3D-fabric (dimensions: length 140 mm, width 90 mm, thickness 0.5 mm, fibre diameter 13 µm) provides a scaffold for hepatocyte immobilization and self-aggregation. Its surface for attachment is about 15 times its projected area which enables high density hepatocyte culture. The oxygenation hollow-fibres (external diameter 630 µm, internal diameter 300 µm) are fixed to the 3D-carrier in a parallel fashion by weaving, spaced at an average distance of 2 mm. In general, this is carried out by folding the matrix material three or more times, making a number of holes in the folded matrix material spaced 2 mm apart by means of a needle, putting the hollow fibres of suitable length through the holes thus obtained, and then again streching the folded matrix material in the direction of the fibres so as to remove the folds, giving a matrix material with the hollow fibres oriented in a unidirectional parallel fashion.

This polyester-polypropylene composite is spiral wound like a swiss roll with the help of an acrylic core (Fig. 3) and placed in a polysulfon dialysis housing (Minifilter, Amicon Ltd, Ireland, ID 1.4 cm, ED 1.7 cm, total length 15.5 cm). The oxgenation hollow-fibres are embedded in polyurethane resin (PUR-system 725 A and 725 BF, Morton International, Bremen, Germany) using dialyzer potting techniques and fitted with gas inlet and outlet endcaps.

The resulting bioreactor is shown in figure 12, with 31 being the housing, 32 being the polyurethane potting, 33 being the extra fibre space inlet and outlet, respectively, 34 being the extra fibre space and 35 being the hollow fibres.

The bioreactor is sterilized by autoclaving (20 min at 121°C). Hepatocyte seeding in the extrafibre space (volume 11 ml, suited for future in vivo experiments in the rat) is realized by injecting the cell suspension via the inlet and outlet ports normally used for dialysate

flow. The same ports are used for medium perfusion after cell immobilization.

1.c *Hepatocyte culture*

Hepatocytes suspended in ice cold Williams'E medium (Gibco BRL Life Technologies, European Division) supplemented with heat inactivated FCS (10%, Boehringer Mannheim), glutamin (2 mM, BDH Laboratory Supplies Ltd.), insulin (20 mlE, Novo Nordisk, Denmark), dexamethason (1 µM) and antibiotic/antimycotic solution (Gibco) at a concentration of 20.10^6 viable cells/ml were injected into two precooled (4°C) dry bioreactors to a final amount of 220.10^6 cells/unit. The cooled bioreactors were integrated into two separate cell perfusion circuits to obtain results in duplicate. This setup was put in a temperature regulated (37°C) cabinet (Stuart Scientific, model SI60, GB) where the bioreactors were clamped onto a rotation device according to figure 11 and connected to culture gas (95% air, 5% CO_2, gasflow: 30 ml/min, 37°C). The reactors 28 were rotated horizontally along their longitudinal axis 29 at 1 revolution/min for a period of 120 minutes to secure an even distribution of the cells throughout the reactor and to accelerate immobilization by entrapment, attachment, and self-aggregation of viable hepatocytes. Every minute the rotation direction was reversed automatically to prevent the connecting tubing from knotting. After this immobilization period an 15 hour intermittent fresh medium waste wash was performed (60 ml) to flush dead and unattached cells out of the reactor, to supply nutrients to and remove toxins from the cell region, and allow the hepatocytes to recover from the isolation procedure. Then, the devices were ready for use.

1.d Hepatocyte function tests

General description

The study included bioreactors with and without hepatocytes, the latter serving as controls. Both groups received identical treatment and monitoring. The hepatocyte function tests were performed under recirculating conditions, perfusing supplemented Williams'E medium (30 ml) through the extrafiber bioreactor space at a flow rate of 5 ml/min. Various parameters were assessed over a period of 4 days, the last day exclusively reserved for protein secretion under serum free conditions. On every day during the first three days a battery of tests was carried out including, galactose elimination, urea synthesis, lidocaine metabolism, and a subsequent 14 hour incubation with supplemented Williams'E medium to evaluate the amino acid metabolism, lactate/pyruvate ratio, enzyme leakage, glucose levels and pH. Every test was preceded by a fresh medium waste wash. Samples collected from the closed loop circuit were snap frozen in liquid nitrogen and stored at -70°C prior to analysis.

Galactose elimination.

D-Galactose (Sigma Chemical Co., St Louis, MO) was administered to the closed loop circuit at a concentration of 1 mg/ml and incubated for 3 hours. Media samples were collected at different time points every day for three days. The galactose concentration was measured at 340 nm (Cobas Bio, Roche, Switzerland) using enzymatic test kits (Boehringer Mannheim, Wiesbaden, Germany, kit no. 1242-73). From this the galactose elimination was calculated.

Urea synthesis from NH_4Cl.

The urea synthesising capacity of the bioreactor system was assessed by incubating 10 mM NH_4Cl for 2 hours. Media samples were collected at different time points every day for three days. Urea was determined colorimetrically at 525 nm (Zeiss UV spectrofotometer) with Sigma Chemical Co. kit no. 535 for urea nitrogen.

Lidocaine metabolism.

Lidocaine-HCl (Sigma) was administered to the closed loop circuit at a concentration of 500 µg/ml and incubated for 1 hour. Media samples were collected at different time points every day for three days. The samples were analysed for lidocaine and three lidocaine metabolites, mono-ethyl-glycine-xylidide (MEGX), 2,6-Xylidine-HCl, glycine-xylidide (GX), by reversed phase high performance liquid chromatography (HPLC). Lidocaine-HCl was obtained from Sigma Chemical Co. and MEGX, Xylidine, GX ,and ethyl-methyl-glycine-xylidide (EMGX) were gifts from Dr. R. Sandberg of Astra Pain Control (Södertälje, Sweden). Sample preparation for the analysis of MEGX, Xylidine and GX involved addition of an 75 µl internal standard solution (EMGX 5 µg/ml in aqua dest) and 150 µl aqua dest. to a 150 µl sample. Analysis of the much higher lidocaine concentrations required a 20-fold dilution of the sample in supplemented Williams'E medium. The isolation of lidocaine and its metabolites was performed by extraction. For this, 150 µl sodium carbonate (0.1 M) and 600µl chloroform were added. After 1 min vortexing and 4 min centrifugation at 8000 rpm the aqueous supernatant was removed and 150 µl aqua dest. and 350 µl HCL (0.1 M) were added to the organic phase. The vortexing and centrifugation procedure was repeated and the supernatant removed. A cooled sample storage compartment kept the residues at 4°C prior to analysis. The mobile phase (0.5 M phosphate buffer, pH 4.5) was pumped at a flow rate of 1.7 ml/min (Perking Elmer 250) and pretreated by a Quard-column (Superspher 60 RP 8, length 10 cm, 4 µm particles, Bischoff Chomatography, Germany). An auto sampler (Gilson Sample Injector model 231, France) injected 50 µl aliquots onto a temperature regulated (55°C, Chrompac Column Thermostat, The Netherlands) HPLC column (Superspher 60 RP 8, length 20 cm, i.d. 4.6 mm, 4 µm particles, Bischoff Chomatography, Germany). Detection was at 198 nm (Schoeffel SF 770 UV-spectrophotometer, Germany) and peak areas

were calculated with the aid of an Olivetti M250 computer utilizing integration software (Chrompac PCI, version 5.12, The Netherlands). The samples were quantified by comparing the peak area ratio of the component of interest to that of the internal standard. Standard curves were obtained for lidocaine (5-80 µg/ml), MEGX (0.5-16 µg/ml), Xylidine (5-80 µg/ml) and GX (1-32 µg/ml) and showed linearity (r=0.996, n=6). The detection limit was 0.4 µg/ml for GX, 0.3 µg/ml for Xylidine, 0.2 µg/ml for MEGX, 0.4 µg/ml for EMGX and 0.5 µg/ml for lidocaine and the retention times were 2.2 min, 2.4 min, 3.2 min, 4.1 min, and 5.8 min, respectively. Column stabilization time was limited to 20 minutes by washing with a phosphate/acetonitril/phosphoricacid buffer (50mM, pH=1.7) and an acetonitril solution (aqua dest.:ACN = 1:1) to remove the chloroform peak.

Amino acid metabolism.

The metabolic turnover of a wide range of amino acids was investigated. The amino acid concentrations were determined by a fully automated precolumn derivatisation with o-phthaldialdehyde (OPA), followed by high-performance liquid chromatography as described in van Eijk HMH, van der Heijden MAH, van Berlo CLH, Soeters PB, Clin Chem 1988; 34: 2510-13.

Lactate/pyruvate ratio. The lactate/pyruvate levels were determined at 340 nm (Cobas Bio, Roche, Switzerland) by enzymatic test kits (Boehringer Mannheim Wiesbaden, Germany, lactate kit no. 149993 and pyruvate kit no. 124982). From this the lactate/pyruvate ratio was calculated.

Enzyme leakage.

Lactate dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were measured by routine clinical analyzers.

Glucose.

Glucose levels were measured using glucose test strips (haemoglucotest 1-44 R, Boehringer Mannheim, Wiesbaden, Germany) and the accessory Reflolux-S readout device.

pH.

The pH was measured by sampling 1 ml of medium with a bloodgas syringe (Marz-175, Sherwood Medical, Ireland) which was determined on a bloodgas analyzer (Radiometer, model ABL 300, Copenhagen).

Protein secretion.

On day four the entire bioreactor culture system was washed with 250 ml supplemented Williams'E medium without FCS and incubated in the same medium. Media samples were collected after 24 hours and dialysed extensively against a 50 mM NH_4HCO_3 solution and frozen dried. The dry residues were reconstituted in such an amount of electrophoresis buffer (Tris-barbital buffer, pH=8.6, ionic strength 0.1) that the culture supernatant was concentrated 20 times.

To visualise the serum proteins secreted by the pig hepatocytes we performed crossed-over immuno-electrophoresis using a polyspecific antiserum to pig serum proteins as described previously (28).

1.e Microscopic examination.

Five day old culture systems were prepared for microscopic examination to determine the orientation of the hepatocytes in the bioreactor.

Light microscopy.

The hepatocytes were fixed by flushing the bioreactor with formaline (4%). After 24 hours the bioreactor was cut open and twelve matrix samples (1 cm^2) were taken from various parts of nonwoven fabric. The samples were

washed in water, dehydrated in graded ethanols, and embedded in paraffine. From this 8 µm thick slices were cut, which were deparaffinated with xylol and coloured with haematoxylin-eosin. The preparations were observed under an Olympus Vamox light microscope (type AHBT3, Tokyo, Japan).

Scanning electron microscopy.

The hepatocyte aggregates from five day old cultures were fixed by flushing the bioreactor with 4% glutaraldehyde in phosphate buffer, pH 7.3 (Fluka Chem A.G., Buchs, Switzerland). The biorector was cut through in the middle and one part was dehydrated in graded ethanols and finally dried in hexamethyldisilizane (Sigma, München, Germany). The cut surface was coated with gold in a sputter coater and observed under a scanning electron microscope (ISI SS40, Japan).

1.f *Statistical analysis.*

An unpaired Student's t-test was used, and $P<0.05$ was considered to be statistically significant. Data were presented as mean ± SEM.

1.g *Magnetic Resonance Imaging (MRI)*

MRI is a non-invasive method for visualizing the liquid flow distribution in for instance a tube, or in the case of the present invention, the bioreactor.

The flow distribution in a cross-section of a small (ID 1.32 cm, volume 11 ml, 46 hollow fibre membranes, diameter acrylic core 0.4 cm) and a scaled-up bioreactor (ID 2.2 cm, volume 33 ml, 138 hollow fibre membranes, diameter acrylic core 0.4 cm) of equal length was investigated. First, the bioreactors were flushed with ethanol and subsequently water to remove air bubbles which can block the medium flow and/or can distort the homogeneity of the magnetic field resulting in a decreased signal intensity. A bioreactor was then placed in a birdcage coil and positioned horizontally in a 6.3 Tesla/20 cm

bore home built spectrometer. Cell free devices were used as the spectrometer was not equipped to support viable hepatocytes. Transaxial flow sensitive MRI's were taken from the middle of the bioreactor using a novel steady state perfusion imaging technique. Briefly, the water signal in a detection slice (width 2 mm, perpendicular to the flow direction) is suppressed. During an in-flow time of 100 ms, part of the slice is refreshed resulting in an increase in signal intensity. So, the higher the flow, the more the detection slice is refreshed, the more the signal intensity will increase. Fluid flow at higher velocities than 2 cm/s will not result in an increased signal, as the detection slice is then completely refreshed. Therefore, the flow was calibrated such that the maximum fluid velocity in most flow channels did not exceed 2 cm/s.

1.h *Alfa-GST assay.*

Toxic serum and hepatocyte viability.

Alfa-GST is released by hepatocytes with a damaged cell membrane, and is therefore a marker for the integrity of the hepatocytes. The liver enzyme alfa-GST was determined *species specific* (rat, pig, human) with an ELISA kit provided by Biotrin, Ireland.

Rats with liver ischemia were treated with a porcine hepatocyte based BAL. Plasma samples were collected in time to determine the rat and pig alfa-GST levels (in one and the same sample).

2. **Results**

2.a Hepatocyte culture.

The study included 22 bioreactors, of which 16 devices (n=8 in duplicate) were used to culture hepatocytes and 6 devices without cells (n=3 in duplicate) served as controls. The results of the hepatocyte function tests in two bioreactors with cells from the same isolation pro-

cedure never differed more than 10%, indicating reproducible cell immobilization and cultivation. The culture system remained sterile throughout the study and no leaking of medium into the lumen of the oxygenation hollow-fibres was observed.

2.b Hepatocyte function tests.

Galactose elimination.

The galactose elimination capacity after incubation for 180 min. with a standard dose of galactose remained constant over a period of three days.

Urea synthesis.

High levels of ammonia play a role in hepatic encephalopathy. Synthesis of urea from ammonia is therefore an important function test. Urea synthesis after a 120 min. incubation with 10 mM NH_4Cl did not vary over a period of three days.

Lidocaine metabolism.

The cytochrome P450 activity of the hepatocytes was assessed by determining lidocaine and its metabolites. The lidocaine elimination and subsequent MEGX and Xylidine production after a 60 min. Lidocaine incubation did not significantly change over a period of three days. Xylidine was the main lidocaine metabolite on the first two days. There was no significant difference in Xylidine and MEGX production on day 3. When looking at individual experiments, lidocaine clearance correlated better with Xylidine than MEGX formation. Porcine hepatocytes did not produce detectable levels of the metabolite GX during incubation with lidocaine for one hour.

Amino acid metabolism.

Table 1 shows the changes in the medium concentration of some amino acids that are relevant for liver function. A

decrease in glutamine concentration was associated with an increased glutamate concentration. Liver metabolism of aromatic amino acids (AAA) was reflected by a decrease in the concentrations of phenylalanine, tyrosine, and tryptophane. Decreased arginine concentrations and synthesis of ornithine are indicative for arginase activity. A decrease in alanine concentration, a precursor of liver gluconeogenesis, was observed.

In addition to table 1, also other amino acids concentrations decreased significantly like, asparagine, glycine, histidine, valine, methionine, isoleucine, leucine, and lysine. Total amino acid metabolism remained stable over a period of three days.

Lactate/pyruvate ratio.

The lactate/pyruvate ratio is an index for the functional state of cellular oxidation and aerobic metabolism. Table 1 shows a drop in the lactate/pyruvate ratio, which was solely due to a decline in the lactate concentration. The lactate/pyruvate ratio of 5 to 7 reflected a stable oxygenation status of the culture system over a period of three days.

Enzyme leakage.

To assess the hepatocyte viability, the appearance of enzyme activity, namely LDH, GOT, and GPT, was determined in the culture medium. LDH release was only significant on day one (Table 1). GOT liberation was significant over the three day period with a downward trend in the average GOT concentration. Low but significant quantities of GPT were released on day 1 and 2.

Glucose.

Glucose levels did not change on the first day of culture (Table 1). A significant decrease in the glucose concentration was observed on day 2 and 3.

pH.

The pH in the studied bioreactor was kept constant (Table 1) by the help of an integrated oxygenator which ensures stable CO_2 partial pressures (32.6±0.4 mmHg, n=8) in the sodium bicarbonate buffered medium.

Protein secretion.

Cultured hepatocytes secrete proteins into their culture medium. A two-dimensional crossed immunoelectrophoresis was performed using an antiserum against pig serum to visualize the different amounts and types of proteins secreted by the hepatocytes cultured in the bioreactor after a 24 hour incubation with supplemented Williams'E medium without FCS. The results are shown in Fig. 6. Each peak represents a different protein. The area under each peak is an indication for the amount of protein secreted. In culture medium from control bioreactors without cells no pig serum proteins were detected (results not shown).

2.c Microscopic examination.

The hepatocytes from the injected single cell suspension reorganized into small irregular shaped aggregates with extensive cell-cell contact (Fig. 14) The aggregates from this five day old culture were immobilized on and entrapped within the polyester fibre framework. Despite high density culturing there is sufficient room between the aggregates for unhindered perfusion of medium to and from the hepatocytes. As the 3D-matrix is relatively empty it has the potential to culture hepatocytes at even higher densities than the present 20.10^6 viable cells/ml. Since the aggregates are so small (one diameter never being larger than 5 cells, mostly 2-3 cells), the hepatocytes function in direct medium contact. Medium flow through this hepatocyte immobilization compartment approximates the in vivo situation where every hepatocyte operates under perfusion conditions and close blood contact.

Examination of 3D-matrix samples taken near the inlet and outlet port and in the middle of the nonwoven fabric revealed that the hepatocytes are evenly distributed in the bioreactor device.

Cell counts in twelve microscopic preparations of the 3D-matrix (dimensions: length 10 mm, width 0.5 mm, thickness 8 µm) of one bioreactor resulted in an average number of 1379±135 (mean±sd) hepatocytes/preparation. One can calculate that if each of the 220.10^6 seeded viable hepatocytes would immobilize within the nonwoven fabric, every preparation should contain about 1400 viable cells. So, on average 98.5% of the hepatocytes were immobilized in this experiment.

Fig. 15 presents a scanning electron micrograph of isolated hepatocytes after five days in culture in the 3D-matrix of the bioreactor. As observed by light microscopy, the hepatocytes from this five day old culture maintain their aggregate configuration and remain immobilized on the polyester fibres. Extensive cell-cell contact between the spherical hepatocytes can be observed.

2.d Magnetic Resonance Imaging

Figures 15A and 15B display the flow distribution in a cross-section of a small (A) and a scaled-up bioreactor (B). The fluid velocity was detected only in the axial direction and ranged from zero (black) to around 2 cm/s (white). When compared with figure 1 several components of the bioreactor can be identified such as, the nonwoven polyester fabric, the oxygenation hollow-fibre membranes, the flow channels, and the acrylic core. The black representation of the nonwoven polyester fabric indicates only that medium flow within the 3D-matrix was not in the axial direction. The perifusion of the fabric in other directions was not investigated. The homogenous distribution of the grey spots demonstrate that all flow channels in both devices were perfused. The shades of grey indicate that the fluid velocity could differ per flow

channel (ranging from 0.5 to 2 cm/s, but mostly around 1.5 cm/s). The arrows in figure 2B show spots of decreased signal intensity as a result of entrapped air bubbles. Spin echo images (not shown) revealed that the size of the air bubbles was much smaller than the resulting distortion. The spectrometer only allowed a horizontal orientation of the bioreactor. Normally, the device is positioned vertically, which facilitates the removal of air bubbles.

Figures 15A and 15B show transaxial flow sensitive MRI's of a small (A, internal diameter 1.32 cm) and a scaled-up bioreactor (B, internal diameter 2.2 cm). The fluid velocity ranged from zero (black) to around 2 cm/s (white). When compared with figure 1 several components of the bioreactor can be identified such as, the nonwoven polyester fabric, the oxygenation hollow-fibre membranes, the flow channels, and the acrylic core. The images of both devices show that all flow channels were perfused. Differences in the fluid velocity of the flow channels can be observed. The arrows in figure B indicate spots of decreased signal intensity as a result of entrapped air bubbles.

2.e Alfa-GST determination

As expected, the rat alfa-GST concentration increased during BAL-treatment. Remarkably, the pig alfa-GST concentration remained constant, indicating that the viability of the porcine hepatocytes in the bioreactor of the invention was not effected by the toxic rat plasma.

3. Discussion

In the prior art, hepatocytes have been cultured in the intraluminal and extrafibre space of hollow-fibre units. The popularity of this concept of cell culturing can be easily understood as it is the simplest way of realising a BAL. These systems however do not meet the essential conditions for optimal mass transfer to and from the

hepatocytes as present in the intact liver. As a consequence, hepatocyte metabolic activity is impaired for the following reasons:

Clinical treatment of hepatic failure requires large scale, high density hepatocyte culture. In many bioreactors this gives rise to the formation of non-physiological hepatocyte pellets. Hepatocytes in the centre of these large aggregates show poor metabolic activity and even possible necrosis due to high gradients as a result of hindered mass transfer of nutrients and oxygen to and carbon dioxide, toxins and cell products from these cells this is in contrast to the in vivo liver where every hepatocyte is in close contact with the blood. Besides, in most bioreactors substrate exchange depends on diffusion which further limits mass transfer compared to the in vivo situation where hepatocytes function under perfusion conditions with corresponding low gradients.

The novel bioreactor of the invention, when used as a BAL addresses the above mentioned requirements for physiological mass transfer. This resulted in a system with the following features:

1. Three-dimensional nonwoven polyester fabric.

Microscopic examination showed that the polyester fibres of the nonwoven fabric provide a framework for high density hepatocyte immobilization (20.10^6 cell/ml) and organisation into small aggregates (one diameter never being larger than 5 cells, mostly 2-3 cells) with room between the aggregates. This allows every cell to operate under in vivo like perfusion conditions and direct medium contact, thereby more closely mimicking physiological gradients. Research on porcine hepatocyte aggregates revealed that such structures maintain many of the cyto-architecture characteristics found in vivo, they survive longer, and show maintained and/or enhanced functional activity compared to monolayer culture. Similar results have been found for our novel bioreactor device which is

based on such porcine hepatocyte aggregates. Microscopic evaluation showed extensive contact between spherical shaped hepatocytes as observed in vivo. Liver specific functions were maintained over a period of three days and the urea synthesizing capacity was doubled compared to monolayer culture, in accordance with Lazar et al.

2. No extracellular matrix materials.

In a previous study (te Velde AA, Ladiges NCJJ, Flendrig LM, Chamuleau RAFM, J Hepatology 1995; 23: 184-192) the functional activity of porcine hepatocytes attached to hydrophilic tissue culture plastic was compared to cells attached to several extracellular matrix constituents: collagen I and IV, laminin, fibronectin, Engelbreth-Holm-Swarm Natrix and in the presence of Matrigel. With the exception of Matrigel, neither of the extracellular matrix substrates enhanced pig hepatocyte function compared to tissue culture plastic. Matrigel has the disadvantage that it is very expensive and moreover, relatively large amounts of murine proteins of tumour origin leak out of the gel and might get into the circulation of the patient. We therefore decided to inject the hepatocyte suspension directly into the dry bioreactor and let the porcine hepatocytes immobilize on the hydrophilic polyester fibres. No prerinsing with medium nor coating with common extracellular matrix materials like Matrigel, collagen or others was performed, resulting in a safer, cheaper and more convenient device.

3. Rapid hepatocyte immobilization.

The hepatocytes are allowed to immobilize for two hours. After the immobilization period dead and unattached cells are flushed out of the bioreactor, hence improving the overall viability of the culture system. Theoretically, the system is then ready for use. Light microscopic examination of a five day old bioreactor revealed that

98.5 % of the seeded viable hepatocytes were present in the 3D-matrix, indicating high immobilization efficiency and limited cell washout in time. The latter result was confirmed by daily light microscopic examination of medium samples from the closed loop circuit, in which cells or debris were rarely observed.

A short preparation time could be an advantage in clinical application, but additional research needs to demonstrate what the effect this limited recovery time has on hepatocyte function. The rapid immobilization can be explained as follows: after injection of the single-cell hepatocyte suspension, the bioreactor is rotated horizontally along its longitudinal axis for two hours. This continuously changes the sedimentation direction of the suspended cells, which allows the hepatocytes to "shop around" the bioreactor space in search for polyester fibres to attach to. The rotation mode drastically enhances the cell-fibre and cell-cell interactions, thereby accelerating attachment and aggregation of viable hepatocytes. Moreover, the optimal oxygenation status of the system further improves the rate of hepatocyte immobilization.

4. Low substrate and metabolite gradients.

On a cellular level low substrate and metabolite gradients in a high density hepatocyte culture can be realized by culturing the hepatocytes as small aggregates inside the nonwoven polyester fabric. This results in hepatocyte culture with sufficient room between the aggregates for unhindered perfusion of all hepatocytes with low medium gradients. When looking at the entire bioreactor, low medium gradients can be obtained by either reducing the perfusion distance between the inlet port and outlet port or by increasing the medium flow rate. Gerlach et al mentioned hereinabove descibe a complicated bioreactor design that realized the former option by culturing the hepatocytes between independently

woven hollow-fibre bundles, among one for medium inflow and another for medium outflow. This allows decentralized perfusion of the cells between these capillaries with low gradients. A technically much simpler solution is the latter option by increasing medium flow rate through the bioreactor. This was feasible in our system as the hepatocytes are cultured inside the 3D-matrix and thereby protected by the polyester fibre network. Stepwise increasing the medium flow rate from 5 ml/min to 15 ml/min did not reveal any signs of shear stress such as, a decrease in hepatocyte functional activity or an increase in enzyme leakage. Uniform flow and distribution of medium to all parts of the 3D-matrix is ensured by numerous channels, which are evenly distributed throughout the bioreactor space. Moreover, these channels also take care of a homogeneous supply of the injected hepatocyte suspension to the 3D-matrix.

5. Decentralised oxygenation and even oxygenation hollow-fibre distribution.

The integrated oxygenator eliminates O_2 and CO_2 gradients along the perfusion direction of the medium. Furthermore, the spiral wound construction (fig. 3) creates a homogeneous distribution of the oxygenation hollow-fibres throughout the bioreactor thereby ensuring every hepatocyte of an oxygenation source within its direct surroundings. This results in an optimal oxygenation of the hepatocytes, which was confirmed by a sharp decrease in the lactate/pyruvate ratio (32), and stable pH indicating constant CO_2 partial pressures in the sodium bicarbonate buffered medium.

6. Biocompatibility.

Biocompatibility has been addressed by constructing the bioreactor of materials that have been FDA approved and withstand the high thermal stress of autoclaving. As far as we know this is the first bioreactor for hepatocyte

culture that can be steam-sterilized. This is biologically much safer than the normally used very toxic ethylene oxide sterilization, because ethylene oxide residues leak out of polymers for weeks on end and may cause sensitisation and allergic reactions in patients.

7. Easy scaling up.

The hepatocyte immobilization compartment is composed of many repetitive units. Each unit is fully capable of supporting hepatocyte function and incorporates an oxygenation hollow-fibre, a channel for medium perfusion, and three-dimensional carrier material. Scaling up to the liver mass needed for clinical application simply implies increasing the number of units, thus increasing the number of windings of the hollow-fibre/3D-matrix composite until the required immobilization capacity has been obtained. The use of standard dialysis housings and potting techniques ensure easy manufacturing of a wide range of bioreactor sizes.

Such scaling-up will not influence the plasma distribution in the bioreactor. This was confirmed by flow sensitive MRI, which showed perfusion of all flow channels in a small and a scaled-up bioreactor. The fluid velocity could differ per flow channel, which is a result of the fact that the bioreactors were hand-made. Industrial production techniques are currently evaluated to solve this.

Also, with the alfa-GST assay mentioned above, for the very first time it is now possible to simultaneously monitor the condition of the liver of the patient and the hepatocytes in the bioreactor. As pig livers are thought to be the hepatocyte source of choice for the years to come this test could be an interesting candidate for monitoring hepatocellular damage during BAL treatment.

A bioartificial liver support system for the treatment of fulminant hepatic failure and as a bridge to

liver transplantation requires large amounts of viable and actively functioning hepatocytes. Pig hepatocytes are considered to be the best alternative, as human hepatocytes are scarcely available and transformed cells may lack critical hepatocyte functions. Pig livers can be obtained from laboratory animals or from the slaughter house, and pig hepatocytes can be easily isolated in large quantities with a simple two-step collagenase perfusion technique.

For clinical application of a bioartificial liver no long-term cultured hepatocytes are advisable, as the metabolic functions of cultured primary hepatocytes decline with time. Therefore, we monitored the culture system only over the first four days after isolation when liver specific functions are highest. The diversity in liver functions doesn't allow a single test to be an indication for hepatocyte functional capacity. For this reason, a battery of tests was carried out to assess the performance of the culture system. The galactose elimination, urea synthesis and amino acid metabolism remained constant over the investigated period of three days, indicating the ability of the bioreactor system to maintain hepatocyte function.

Lidocaine clearance is an indication for cytochrome P450 activity and is considered the critical function that must be provided by a successful BAL. Lidocaine clearance was maintained over three days, thus demonstrating stable P450 activity by the bioreactor cultured hepatocytes. In one single experiment P450 activity was sustained over 14 days with a gradually decreasing trend in the second week to 70% of the initial activity. To exclude that lidocaine clearance was caused by evaporation, adsorbtion or unchanged uptake by hepatocytes, biotransformation of lidocaine was investigated by detecting the metabolites MEGX, Xylidine, and GX, known to be synthesized in man. MEGX is reported to be the main lidocaine metabolite in man and in porcine hepatocytes cultu-

re. In contrast, not MEGX but Xylidine was the main lidocaine metabolite on the first two days of culture in this study. Furthermore, porcine hepatocytes did not produce detectable levels of the metabolite GX. In summary, Xylidine and MEGX synthesis confirmed cytochrome P450 activity as demonstrated by the lidocaine clearance. The biotransformation of lidocaine in porcine hepatocytes differed from what has been observed in man.

The concentration of LDH, GOT, and GPT, used as a marker of cell membrane integrity, decreased rapidly over the investigated period. This drop in enzyme levels probably indicates the recovery of the cultured hepatocytes from the harmful effects of the enzymatic cell isolation technique. The GPT concentrations in our culture system were very low compared to the LDH and GOT levels, which was also observed in the prior art. Therefore, we conclude that the GPT is a poor indicator of porcine hepatocyte membrane integrity and should better be left alone. The most sensitive marker in this study was GOT.

Another important liver function is the protein secretion, which was investigated in the hepatocyte bioreactor on the fourth day of culture. The culture system was able to secrete various proteins as visualized by crossed-over immunoelectrophoresis, each peak representing a different serum protein.

In conclusion, the invention provides a novel bioreactor configuration which ensures maintenance of various liver specific functions at high density hepatocyte culturing. This, together with its ease of handling, manufacturing, and scaling up, makes the system an attractive candidate for short term support of patients in hepatic failure.

Table 1:

Results of a 14 hour incubation (every day for three days) of 220.10^6 bioreactor cultured hepatocytes with supplemented Williams'E medium concerning changes in amino acid concentrations, lactate and pyruvate concentrations and lactate/pyruvate ratios, enzyme leakage, glucose concentrations, and pH.

Evaluation	Unit	t = 0*	day 1	day 2	day 3
Glutamate**	µM	402.9±6.6	851.3±80.4	971.6±62.6	1038.2±94.6
Glutamine	µM	1893.0±47.1	881.4±106.6	809.3±119.0	784.0±124.0
Phenyl-alanine	µM	155.1±2.2	62.1±6.2	59.1±6.7	68.2±5.03
Tyrosine	µM	181.7±2.3	58.0±12.6	51.8±18.2	50.6±14.0
Tryptophan	µM	50.6±0.7	20.6±4.1	13.5±3.7	11.8±1.5
Arginine	µM	306.9±9.3	15.0±2.0	15.0±4.4	18.4±6.1
Ornithine	µM	28.2±3.8	231.5±24.1	229.8±27.7	250.4±28.3
Alanine	µM	1088.4±20.9	408.8±89.0	457.7±82.0	424.6±64.4
Lactate***	mM	1.44±0.02	0.34±0.07	0.26±0.06	0.27±0.05
Pyruvate	mM	0.08±0.004	0.05±0.01	0.05±0.01	0.05±0.004
Lact/Pyr ratio	-	18.0±0.8	6.7±0.8	5.4±0.5	5.6±0.8
LDH***	U/L	14.3±0.9	35.2±3.5	21.0±2.7	14.8±1.3
GOT	U/L	4.2±0.2	169±40	120±41.9	93 ± 34
GPT	U/L	0.95±0.2	2.1±0.3	1.7±0.2	1.4±0.2
Glucose***	mM	12.0±0.1	12.4±0.7	10.9±0.4	9.6±0.6
pH***	-	7.459±0.025	7.360±0.020	7.394±0.010	7.401±0.011

* The zero point sample (n=8) was collected just after an extensive waste wash with supplemented Williams'E medium.
** mean of 6 experiments in duplicate ± SEM.
*** mean of 8 experiments in duplicate ± SEM.

Example II - In vivo results

The abovementioned BAL was tested with an in vivo rat model.

The used population of rats was divided in three groups.

a. Reference group 1: liver ischemia (LIS) rats given only an infuse.

b. Reference group 2: LIS rats connected to the entire BAL system, but without hepatocytes. This reference is carried out to study the influence of plasmapheresis (possible negative effect) and the large volume of the extracorporal circulation (possible positive effect through dilution of toxins) on the survival.

c. LIS rats connected to the BAL with pig hepatocytes.

Conclusions:

The rats were tested for survival. No difference in survival was found between reference groups 1 and 2 (5.9 ± 2.0 hours and 5.5 ± 1.6 hour respectively, n = 8). The extracorporal circuit therefore has no significant influence on the survival. Compared to the references, LIS rats treated with a BAL system comprising hepatocytes lived twice as long (11.0 ± 2.2 hours, n = 5). This is a remarkable result, not achieved in the prior art, in particular because

a. The model used is very aggressive. Apart from the fact that the rat liver has been completely taken out, toxins leak from the ischemic liver into the rat's circulation, which further detrimentally effects the condition of the tested animal. This is also in accordance with the clinical situation.

b. The rat has not been treated with hepatocytes from its own species, but with pig hepatocytes. This is also in accordance with the clinical situation.

C L A I M S

1. Solid support for use in cell cultivation in vitro, comprising a 3D-matrix material and hollow fibres being permeable to at least gaseous oxygen and/or gaseous carbon dioxide.

2. Solid support according to claim 1, wherein the 3D matrix material is a material providing a high-surface area substrate, the effective surface of which is from 10 to about 100 times the area of the same projected onto a plane surface, comprising a physiologically acceptable network of fibres having a porosity from 40 to about 95% and a pore size of the order of 10 µm to 100 µm, or an open-pore foam structure with a pore size from about 10 µm to 100 µm, the overall hight of the matrix being of the order of 50 µm to about 2000 µm, said matrix being in the form of a highly porous, non-woven sheet or mat, and said hollow fibres are made of a hydrophobic material and have an outer diameter of 0.1 - 1.0 mm

3. Solid support according to claim 1 or 2, in which the hollow fibres are evenly distributed throughout the 3D matrix material.

4. Solid support according to claim 3, in which the fibres are arranged in an essentially parallel fashion, wherein the distance between the individual hollow fibres is between 0.5 mm - 5 mm, preferably between 1 - 3 mm, more preferably about 2 mm.

5. Solid support according to any of the claims 2 - 4, wherein the fibres are attached and/or physically bonded to the matrix sheet or mat by weaving them into the matrix sheet or mat, glueing them or sewing them onto the matrix sheet or mat, or by bonding them to the matrix sheet or mat by means of ultrasound.

6. Solid support according to any of the claims 1 - 4, wherein the hollow fibres are present as a hollow fibre containing sheet.

7. Solid support according to claim 6, the 3D-matrix material being provided as a separate sheet or mat or a sheet or mat attached to and/or laminated onto the fibre containing sheet.

8. Biological reactor for the cultivation and/or maintenance of living cells, said reactor comprising a wall, surrounding a space, and comprising a solid support according to any of the claims 1 - 7, said reactor optionally being provided with at least one gas inlet and at least one gas outlet operably connected to the hollow fibres of the solid support and optionally at least one liquid inlet and one liquid outlet operably connected to the extra fibre space.

9. Biological reactor according to claim 8, wherein the solid support is present in the reactor in the form of one or more rolled up or folded up sheets or mats, or two or more stacked up sheets or mats.

10. Method for culturing and/or maintaining living cells, wherein said cells are introduced into a reactor according to claim 7 or 8 and kept in said reactor under physiologically acceptable conditions by supplying gaseous oxygen or an oxygen containing gas through the gas inlet operably connected to the hollow fibres, optionally by supplying nutrient-containing liquid medium through the liquid inlet operably connected to the extra fibre space, and by keeping the cells at a physiologically acceptable temperature.

11. Method according to claim 10, wherein the cells are chosen from plant or animal derived adherent tissue cells and cell lines derived therefrom, such as hybridoma cells.

12. Method according to claim 11, wherein the cells are chosen from human or animal derived liver cells, chosen from primary hepatocytes, immortalized liver cells, liver cell transformants, hepatoma cells and hepatoblasts, as well as cell lines derived therefrom.

13. Method according to claim 11 or 12, wherein, after the cells have been introduced into the bioreactor, the adherent cells are immobilized on the solid support present in said bioreactor by a method comprising rotating the reactor around an internal or an external longitudinal axis, preferably around an external axis using an apparatus as shown in figure 11, during a period of time suitable for allowing the cells to adhere to the solid support.

14. Bio-artificial liver, comprising a biological reactor according to claim 8 or 9.

15. Bio-artificial liver according to claim 14, further comprising human or animal derived liver cells, chosen from primary hepatocytes, immortalized liver cells, liver cell transformants, hepatoma cells and hepatoblasts, as well as cell lines derived therefrom, said liver cells being maintained in the biological reactor by means of a method according to claim 11 or 12.

16. Bio-artificial liver according to claim 15, said liver cells being primary human or pig hepatocytes.

17. Bio-artificial liver according to claim 16, said primary human or pig hepatocytes being subjected to cryopreservation before use.

18. Method for treating liver disorders, comprising supporting and/or replacing the liver function of a patient by the use of a bio-artificial liver according to any of the claims 13 - 16, operably connected to the blood circulation of said patient.

****

Fig-1

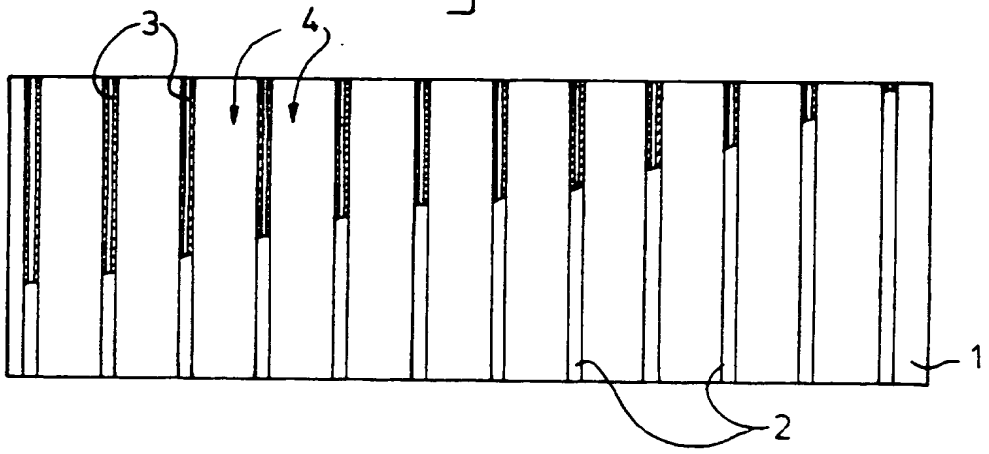


Fig-2

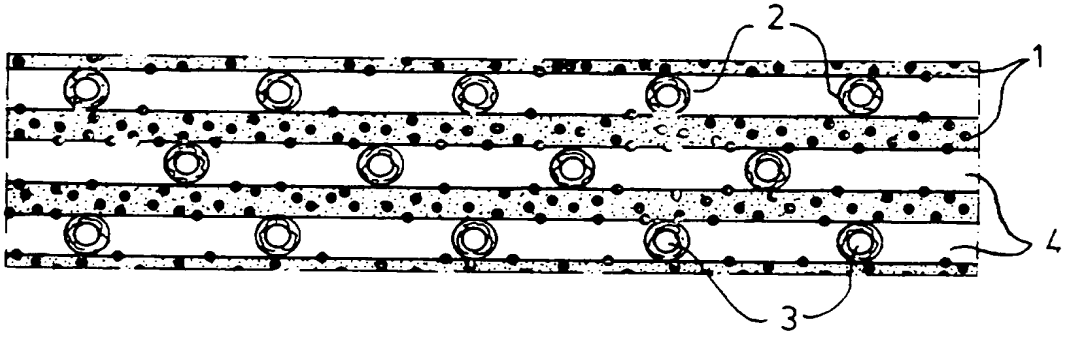


Fig-3

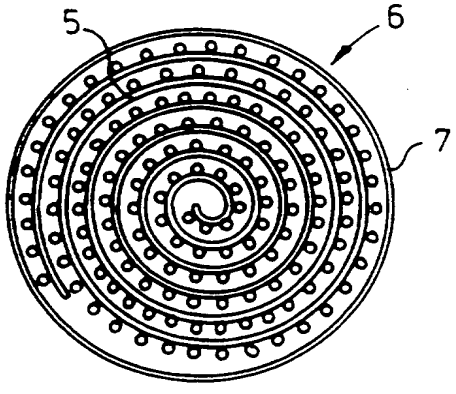


Fig-4

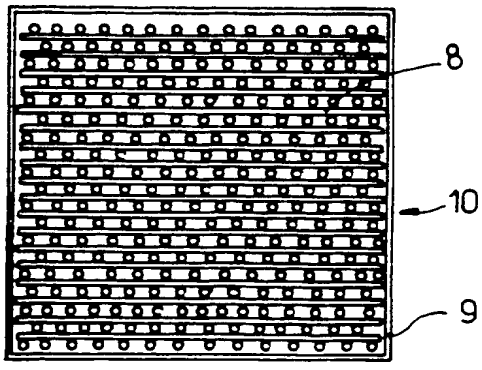


Fig-6

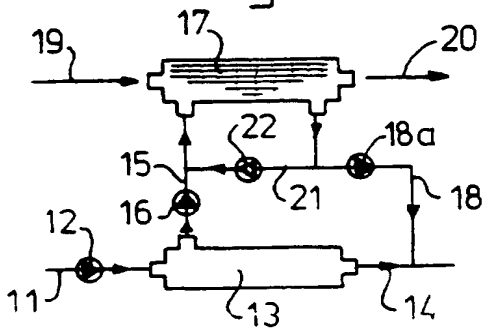


Fig-5

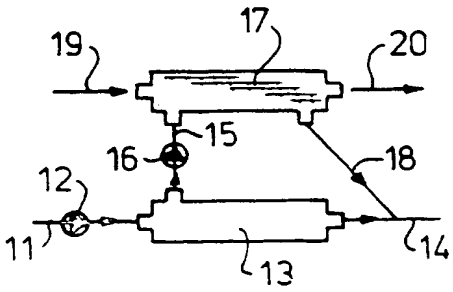


Fig-7

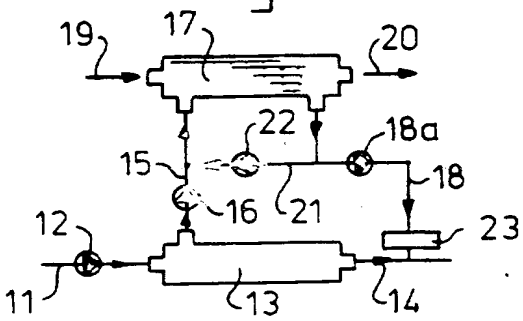


Fig-8

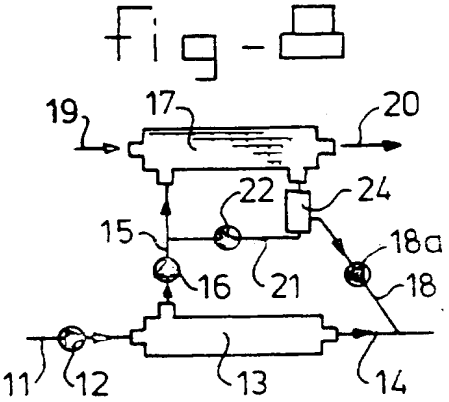


Fig-9a

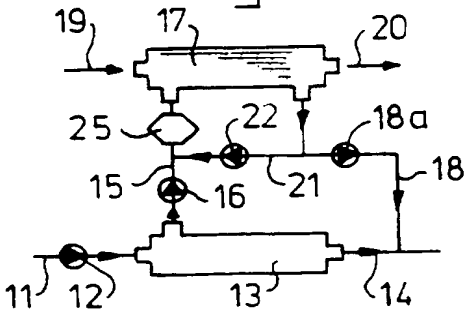


Fig-9b

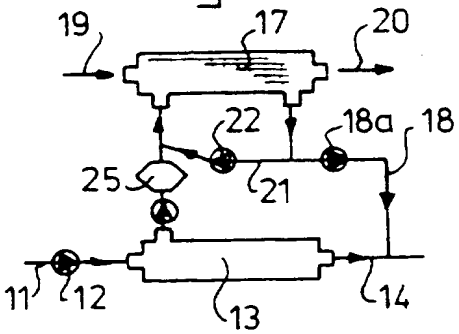


Fig-10

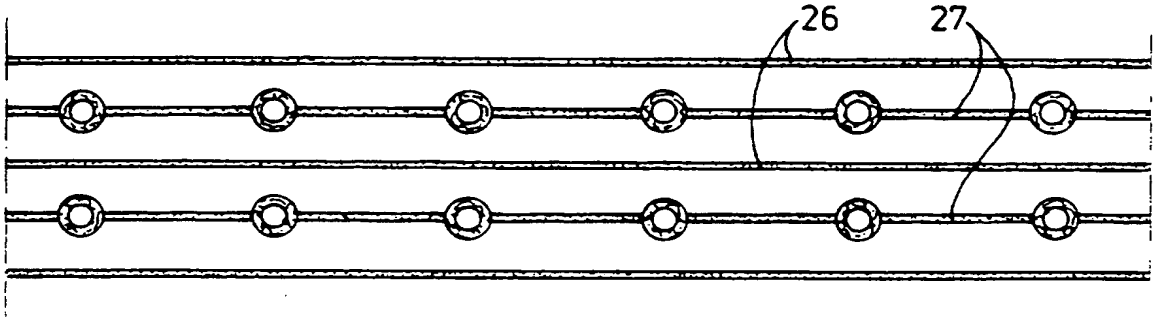


Fig-11

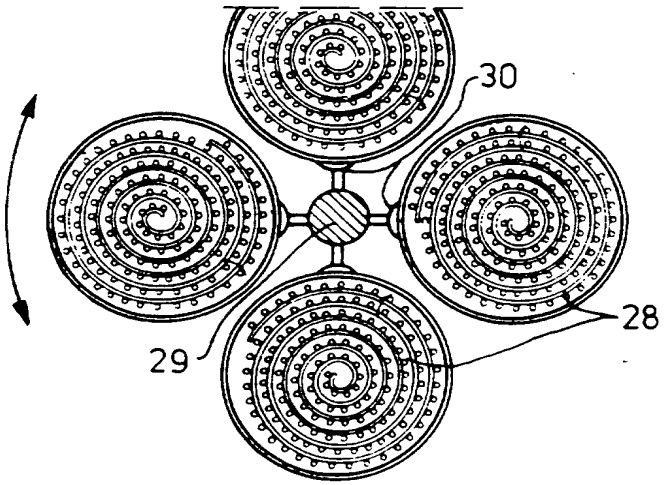


Fig-12

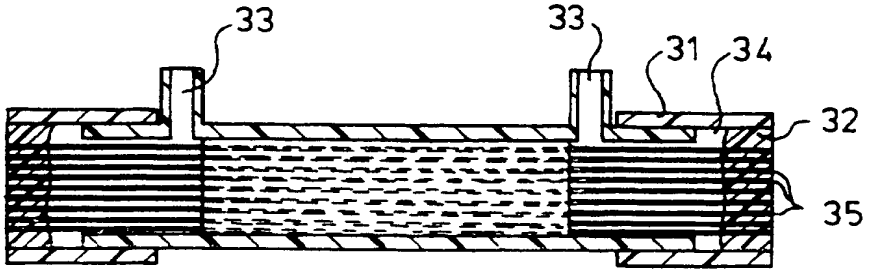


Fig-13

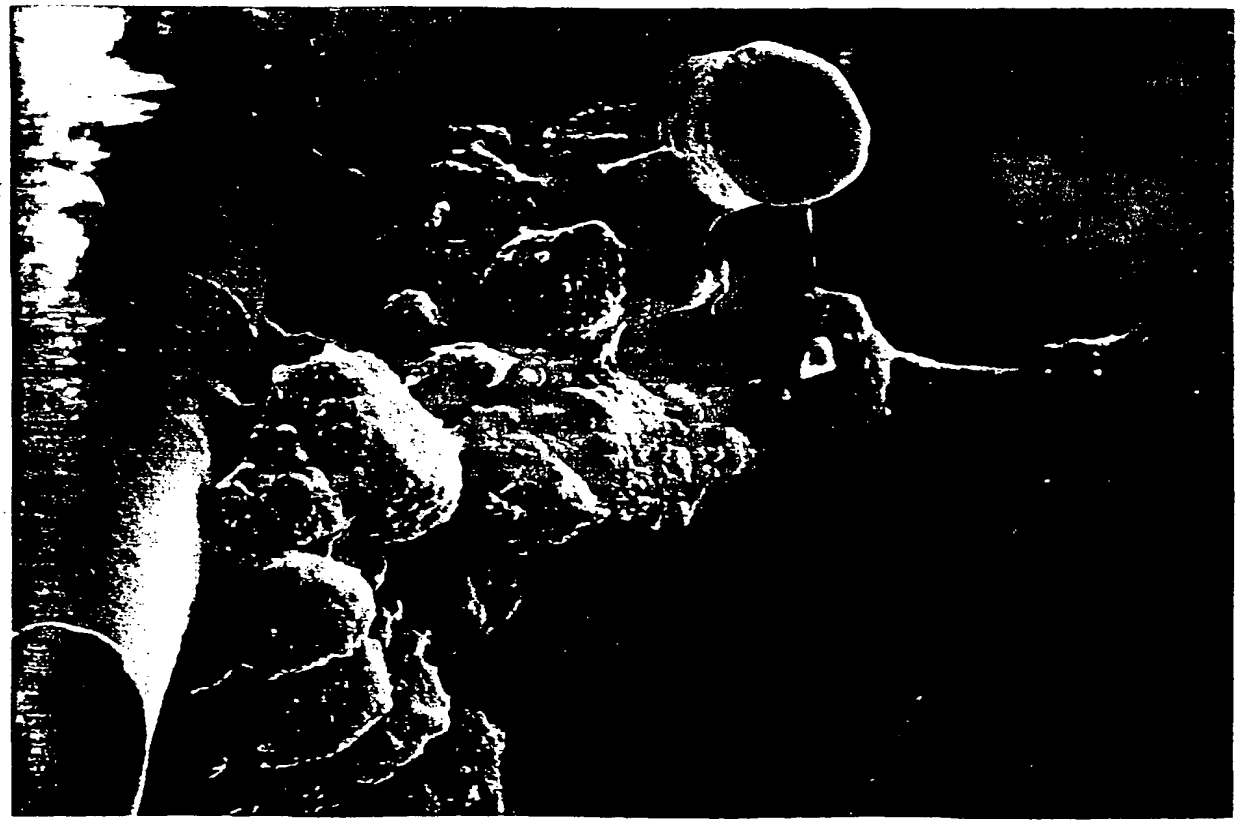
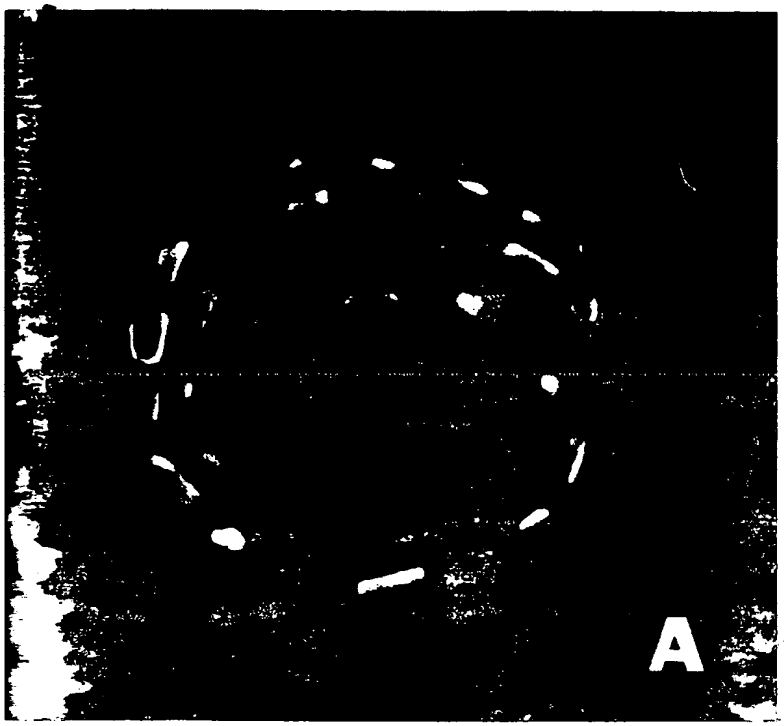
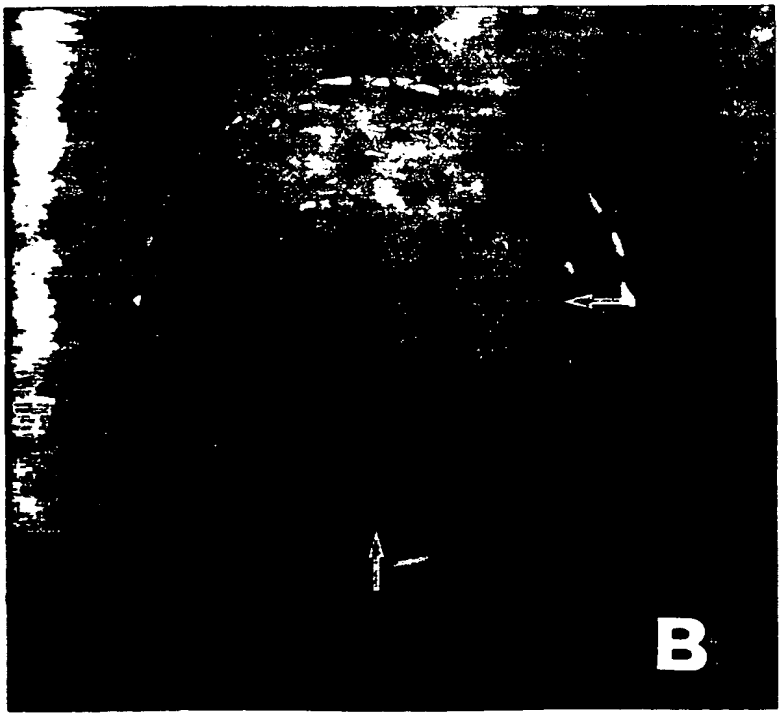


Fig-14

fig-15





INTERNATIONAL SEARCH REPORT

Internatior Application No
PCT/NL 96/00389

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00 C12M1/40 A61M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5 262 320 A (STEPHANOPOULOS GREGORY ET AL) 16 November 1993 see abstract; claims; figures 3,4 see column 9, line 67 - column 10, line 51 ---	1,8, 10-12 1-11
X Y	DE 42 18 917 A (SCHMITZ KLAUS PETER DR ING HAB ;BEHREND DETLEF DR ING (DE); DITTRI) 16 December 1993 see the whole document ---	1,3,5,8, 10,11 1-11
X Y	US 4 537 860 A (TOLBERT WILLIAM R ET AL) 27 August 1985 see column 6, line 15 - line 43; claims; figures see column 12, line 33 - line 50 see column 5, line 14 - line 50 --- -/--	1,3,8, 10-13 1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
19 February 1997	05. 03. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Coucke, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 16058 A (FULLER JESS PAUL ;CLAYSON TONY (GB); KNIGHTS ANTHONY JAMES (GB)) 21 July 1994 see claims; figures	1
X Y	EP 0 365 313 A (KIRIN BREWERY) 25 April 1990 see column 13, line 4 - line 13; claims; figures	1,3,4,8, 11,13 1-11
P,X Y	US 5 510 262 A (STEPHANOPOULOS GREGORY ET AL) 23 April 1996 see abstract; claims; figures 3,4 see column 9, line 35 - column 10, line 47	1,8, 10-12 1-11
A	DE 43 22 746 A (BADER AUGUSTINUS DR MED) 12 January 1995 see claims; figures	1
Y	EP 0 356 785 A (ENDOTRONICS INC) 7 March 1990 see claims; figures	1-11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 96/00389

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5262320	16-11-93	US-A-	5510262	23-04-96
DE-A-4218917	16-12-93	NONE		
US-A-4537860	27-08-85	CA-A-	1210352	26-08-86
		DE-A-	3377800	29-09-88
		EP-A-	0113328	11-07-84
		JP-C-	1837137	11-04-94
		JP-A-	59118080	07-07-84
WO-A-9416058	21-07-94	AU-A-	5710394	15-08-94
		GB-A,B	2289678	29-11-95
EP-A-0365313	25-04-90	JP-C-	1753319	23-04-93
		JP-A-	2109966	23-04-90
		JP-B-	4039990	01-07-92
		CA-A,C	2001113	20-04-90
		DE-D-	68908835	07-10-93
		DE-T-	68908835	14-04-94
		US-A-	5057428	15-10-91
US-A-5510262	23-04-96	US-A-	5262320	16-11-93
DE-A-4322746	12-01-95	AU-A-	7457994	06-02-95
		WO-A-	9502037	19-01-95
		EP-A-	0708823	01-05-96
EP-A-0356785	07-03-90	US-A-	5079168	07-01-92
		AU-B-	622921	30-04-92
		AU-A-	3924089	15-02-90
		CA-A-	1315232	30-03-93
		DE-T-	68907153	20-01-94
		JP-A-	2107183	19-04-90
		US-A-	5416022	16-05-95

INTERNATIONAL SEARCH REPORT

Inte[illegible]ional application No.
PCT/NL 96/00389

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found muluple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invenuon first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/NL 96/ 00389

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claim 18 is directed to a method of treatment of the human/animal body, the search ahs been carried out and based on the alleged effects of the bio-artificial liver.